CHARACTERIZATION OF FACTOR VIII INHIBITOR BY-PASSING ACTIVITY OF FACTOR IX CONCENTRATES

By

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ABSTRACT

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Hemophilia A is a familial bleeding disorder associated with an abnormal Factor VIII molecule. Bleeding episodes are treated by infusion of antihemophiliac globulin concentrate or cryoprecipitate. Between 5-18% of hemophiliacs develop an antibody to Factor VIII which makes such treatments ineffective. Infusion of Factor IX concentrates may provide a possible solution to this problem.

This study was designed to examine the mechanism responsible for Factor VIII inhibitor by-passing activity (FEIBA) of Factor IX concentrate. A modified activated partial thromboplastin time was designed to assay for FEIBA. The procoagulant and coagulant proteins used in the assay were obtained from the prothrombin complex by biochemical fractionation. In our in vitro system, FEIBA proved dependent on the interaction of Factor X, IIa and V.

Further attempts were made to correlate amidolytic activity of FEIBA with the clotting activity utilizing a synthetic chromogenic peptide substrate, [H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride].

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INTRODUCTION

Classic hemophilia, or hemophilia A, is an inherited, sex-linked bleeding disorder. It exhibits decreased clotting activity due to a deficiency of a specific plasma protein, Factor VIII, which is necessary for normal hemostasis. Clinical manifestations of the disorder include deep tissue bleeding and the residual effects of bleeding. Current treatment of a hemorrhagic episode includes infusion of cryoprecipitate, a by-product of whole blood fractionation, or infusion of lyophilized Factor VIII concentrate, prepared from the plasma of blood donors. However, 7-18% of individuals with hemophilia A develop an inhibitor (an antibody which acts specifically against Factor VIII clotting activity) which makes the standard form of treatment ineffective.

The most efficacious treatment of a bleeding episode in the hemophiliac with an inhibitor, is the infusion of prothrombin complex concentrates. The exact mechanism of the coagulant effect of these concentrates is unknown.

It is the purpose of this thesis to characterize the mechanism of the prothrombin concentrates. In order to approach this problem it was necessary to: 1) isolate and

purify procoagulant and coagulant proteins; 2) devise an assay for evaluating Factor VIII inhibitor by-passing activity (FEIBA); and 3) utilize *in vitro* clotting studies, chromatographic techniques and synthetic substrate assays to analyze the precise mechanism.

The Clotting Factors

Observations of an enzymatic activity involved in clotting date back to 1872 when Schmidt (1) used the term "fibrin ferment" or "thrombin" to describe the material precipitated from serum, not from fresh blood. Morowitz (2), in his classic theory on blood coagulation in 1905, describes thrombokinase and thrombin as two active enzymes in the blood clotting scheme. During the next fifty years, the discovery of Factor V (3), Factor VII (4), and Factor X (5) and the discovery of their role in the conversion of prothrombin to thrombin lent support to Morowitz's theory. Prothrombin, which was merely a hypothetical substance in the early 1900's, was investigated extensively and by the early 1960's Seegers (6) and others (7-9) had elucidated the biochemistry of prothrombin. The study of the coagulation protein interactions led to a hypothetical scheme, proposed by Macfarlane (10), of the pathophysiology of the clotting activity from surface contact to fibrin formation. Davie and Ratnoff (11) also proposed at the same time as Macfarlane the sequential activation of the clotting proteins in a chain-like manner. This is known as the "cascade" or "waterfall" hypothesis.

Figure 1 shows a modified cascade scheme which also includes an alternate pathway referred to as the extrinsic pathway, as well as feedback reactions.

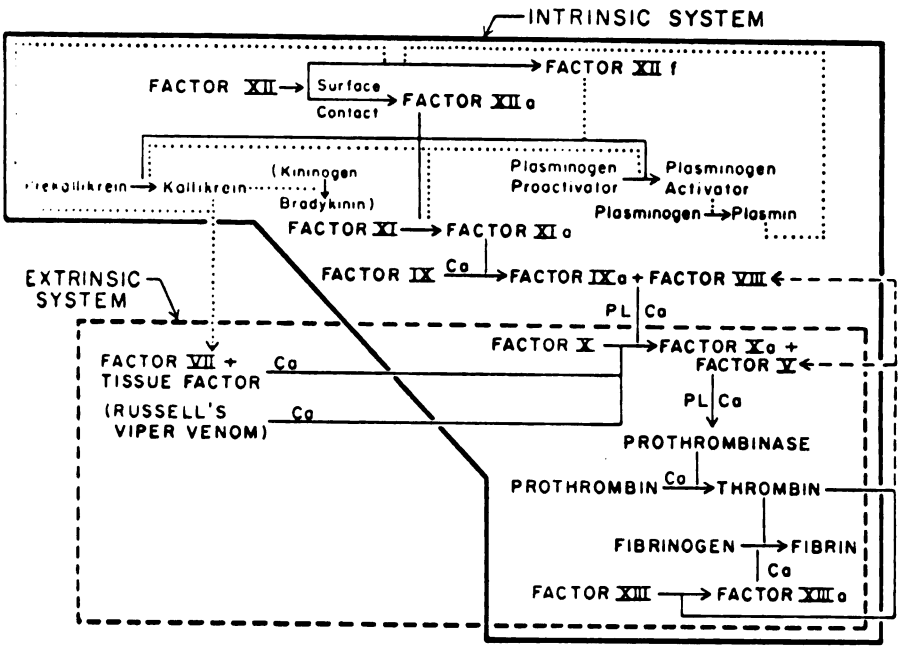

FIGURE 1. A Modified Cascade Scheme For Blood Coagulation. (12).

There is another theory of blood coagulation proposed by Seegers (13) which involves specific terminology relating the clotting proteins to the prothrombin molecule (prothrombin derivatives). At the present time, with modification of both theories and with more extensive characterization of the clotting factors, a more current

scheme of the protein - protein interactions can be postulated.

Prothrombin and Thrombin

The complete biochemistry of bovine prothrombin and its activation is now well known. Bovine prothrombin is a glycoprotein with a single polypeptide chain (molecular weight 70,000 daltons). The complete amino acid sequence of bovine prothrombin is shown schematically in Figure 2. The prothrombin molecule can be divided into three main parts: 1) Prothrombin Fragment 1 which is one activation product; 2) Prothrombin Fragment 2, another activation product; 3) and the remaining portion which constitutes thrombin after the appropriate activation.

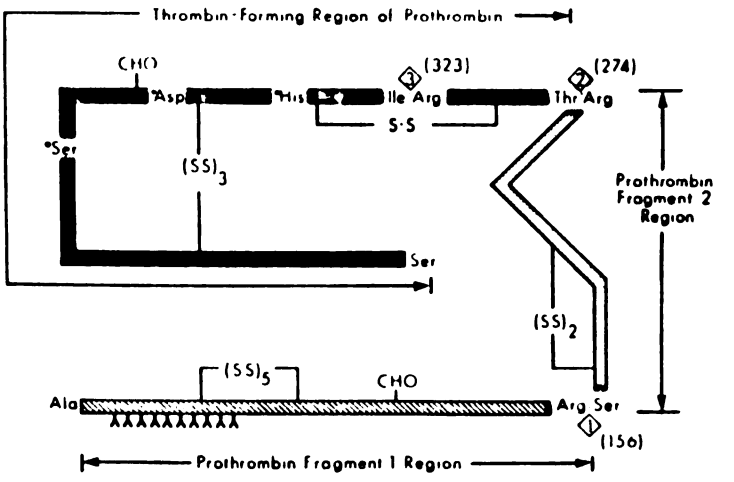

FIGURE 2. Schematic Illustration Of Bovine Prothrombin (14)

Human prothrombin has also been fully characterized (15) and the amino acid sequence is known. Human prothrombin is also a single chain polypeptide with a molecular weight of 72,000 daltons. Recent purification procedures of human prothrombin are included in the work of DiScipio, et al (16). There are some differences in the amino-terminal region of the bovine prothrombin molecule as compared to the human prothrombin molecule. The human prothrombin molecule has a threonine residue in position 3 where the bovine prothrombin has a lysine residue. In position 4, a glycine is present in the bovine species; the human species omits a residue at this position. The purpose for the omission is to allow greater homology with other

human vitamin K dependent proteins. Although minor differences are present among the various clotting proteins, their similarities are quite significant.

The conversion of prothrombin to thrombin is a rapid process dependent on the interaction of Factor Xa, Factor V (Va), lipid surfaces and calcium ions. Factor Xa is a proteolytic enzyme responsible for the cleavage of two peptide bonds in the prothrombin molecule. Factor V, a labile plasma component, binds II, Xa and phospholipid. The lipid surface increases the association between Factor Xa and prothrombin and is made available from platelet or damaged cell membranes. Calcium ions are necessary for the binding of Factor Xa and prothrombin binding to the lipid surface. Figure 3 schematically illustrates the conversion of prothrombin to thrombin (17).

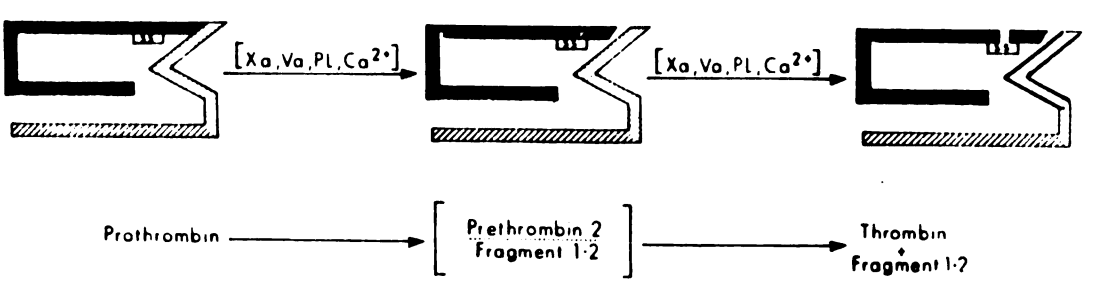

FIGURE 3. The Conversion Of Prothrombin To Thrombin (17)

When prothrombin is activated in the presence of antithrombin III, an additional pathway is reported (18) in which the zymogen is converted to a precursor (P_3) with a

molecular weight of 37,000 and fragment $F_{A\ B}$. This takes place before thrombin generation.

A slower activation of prothrombin occurs when high concentrations of Factor Xa are reacted with Factor II in the presence of 25% sodium citrate (19).

Since the zymogen itself is devoid of biological activity, assay methods are based on the measurement of thrombin formation. The first assay was a one stage procedure developed by Quick (20) in 1935 and modified by Link and Shapiro in 1949. The two stage assay first used by Warner, Brinkhaus, and Smith (21) in 1936 involves the addition of tissue extracts and calcium ions to Factors V, VII, X and prothrombin. This results in optimal thrombin formation. At specified intervals, aliquots of the reaction mixture are removed and added to a standard amount of fibrinogen. Clotting times are recorded. Clotting times of fibrinogen are plotted against the time at which the samples were removed. Clotting times are converted into thrombin units according to appropriate calibration curves. One unit of prothrombin yields one unit of thrombin.

Thrombin

Thrombin is a serine protease that catalyzes the cleavage of specific arginyl and lysyl bonds. It is present in the blood as a result of the proteolytic conversion of prothrombin. Thrombin is less negatively charged than prothrombin and was first obtained as a highly purified

protein in 1955 (22). The molecular weight of bovine thrombin is reported as 33,700 daltons (23) as well as 40,000 daltons (24). The amino acid sequence of bovine and human thrombin has been characterized (25, 26). Very few differences exist between the two species.

Thrombin is responsible for the following actions: 1) conversion of fibrinogen into fibrin; 2) activation of Factor XIII; 3) initiation of platelet secretion and aggregation; 4) reactions with plasma protease inhibitors and 5) activation of Factor V and Protein C.

Thrombin can be separated into three components: alpha, beta and gamma thrombin. Human alpha thrombin is a double chain polypeptide with a molecular weight of 36,600 daltons. It is the form primarily responsible for clotting activity. Alpha thrombin also possesses esterase activity. Beta and gamma thrombin do not exhibit significant clotting activity. All three forms are inhibited by alpha-tosyl-L-lysylchloro-methyl ketone.

Thrombin, whether human or bovine, can undergo autolytic degradation. The degraded species lack clotting activity but exhibit the esterase activity.

Thrombin may be assayed enzymatically utilizing chromogenic peptide substrates. S2160 (Ortho) is sensitive for the determination of thrombin, but is also sensitive to other proteases such as trypsin, papain, and brinase. A more newly developed synthetic peptide is [H-D-phenylalanyl-

L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride], or S2238 which is more sensitive and more soluble than S2160. S2238 is a small peptide with the chromophore attached to the N terminal amino acid. The rate of p-nitroanaline formation is proportional to the enzymatic activity of thrombin.

Factor X

Factor X, also known as Stuart Prower factor, is a plasma glycoprotein and a major proenzyme activated during blood coagulation. Factor X, along with Factors II, VII and IX, are dependent upon vitamin K for their biosynthesis. These coagulant proteins can be absorbed out of the plasma by barium and aluminum salts. Seegers (27) refers to Factor X as autoprothrombin III. Aronson's (28) findings support evidence of a separate origin for Factor X due to the presence of only 1/3 similar amino acid sequences for Factor X as compared with prothrombin. The remaining 2/3 are unique for each molecular species. Later studies (29) report similarities between Factor X and prothrombin as follows: 1) both require Ca^{++} and phospholipid for physiological activation; 2) both have similar amino-terminal amino acid sequences; 3) both are serine esterases with common amino acids at the active site and 4) both contain similar vitamin K dependent calcium binding regions.

Bovine Factor X has been successfully purified using various isolation procedures. (30, 31, 32, 33) It is a glycoprotein (molecular weight 55,000 daltons) composed of two polypeptide chains held together by disulfide bonds. It is also reported to exist as a single polypeptide chain (34). Jackson (35) isolated two glycoprotein variants of bovine Factor X, which differed in carbohydrate composition. Both, however, contained two polypeptide chains. The amino acid composition of the heavy chains of the Factor X molecule is similar to that of thrombin with the exception of three amino acids: lysine, threonine and alanine.

Purification of human Factor X has been more difficult to achieve. Procedures have included preparative isoelectric focusing (36), purification utilizing DEAE cellulose (37) and hydroxyapatite (38). A unique approach has been through isolation of Factor X utilizing blue dextran agarose (BDA) affinity chromatography (39). This purification process yields a homogeneous Factor X (molecular weight 69,000 daltons). Like bovine Factor X, it is composed of a heavy (50,000 daltons) and light (13,000 daltons) chain. DiScipio (40) has characterized human Factor X. He reported a molecular weight of 60,000 daltons. Schematically, Factor X is illustrated in Figure 4. The heavy chain of Factor X (solid black line) contains the active site serine, aspartate and histidine residues.

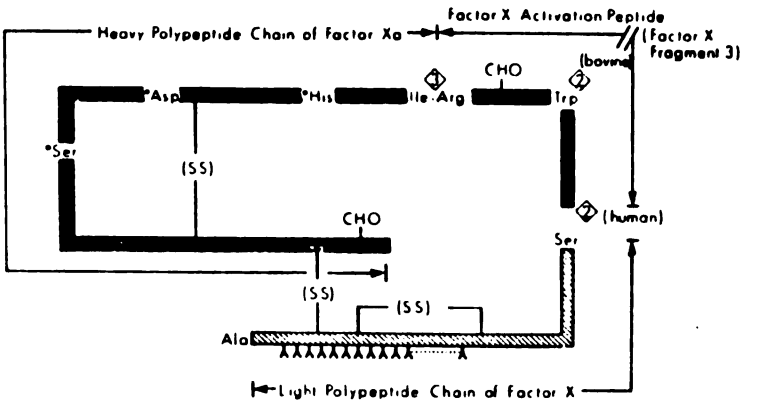

FIGURE 4. Schematic Representation of Factor X (41)

Kosow (42), who purified human Factor X by a combination of DEAE-sephadex, Heparin Sepharose, and hydroxyapatite, achieved a final human Factor X product with a molecular weight of 75,000 daltons.

Factor X can be activated to a serine protease, Xa, via the extrinsic pathway by tissue factor and Factor VII (43,44), or through the intrinsic pathway by Factor IXa and Factor VIII in complex with phospholipid and calcium (45). Factor X can also be activated by trypsin (46, 47), papain (48), or a protease from Russells viper venom (49, 50, 51). Formation of Factor Xa involves cleavage of a specific arginyl - isoleucine peptide bond in the heavy chain of the precursor protein (52). The esterase and coagulant activities of activated Factor X are variably inhibited by diisopropylfluorophosphate (53). Activated Factor X is also inhibited by antithrombin III in a reversible reaction (54)

following second order kinetics. In the absence of heparin, the rate of inhibition is slow.

The qualitative clotting assay for Factor X involves a modified one stage prothrombin assay in which Russell's viper venom is added to Factor VII-X deficient plasma. Russell's viper venom is sensitive to extraneous Factor X but not to Factor VIII. The appropriate lipid and calcium chloride are added to complete the assay.

Factor IX Concentrates

Factor IX concentrates, also referred to as prothrombin complex concentrates (PCC), have been used since 1959 for the treatment of hemorrhagic disorders associated with Christmas disease (55) and liver disease (56). Clinical use of the concentrates was further investigated by Tullis et al (57) who reported therapeutic effectiveness in patients with acquired or hereditary deficiencies of Factors II, VII and X.

The only commercial products available from 1960 to 1968 were made by the French National Transfusion Center or by British laboratories (58). The first commercial concentrate produced in the United States was developed by Cutter Laboratories of Berkeley, California in the late sixties. This concentrate, Hemoplex, which also contained Factors II, VII and X was first reviewed by Hoag et al (59).

Since that time, additional commercial concentrates have been developed. A study by Menache (60) reported 23

different Factor IX concentrates produced by eighteen laboratories. These concentrates vary in several ways including starting material, volume used to prepare one batch, adsorbent used, and the presence or absence of heparin in the final product.

Table 1: A summary of the major and minor procoagulants and coagulants in Factor IX concentrates.

Major	Minor	Miscellaneous
Factor II	IIa	Antithrombin III
Factor X	Xa	Phospholipid
Factor IX	IXa	
Factor VII	VIIa	
Protein C (?)	XII	
	XI	
	VIII	
	V	

The Use of Factor IX Concentrates to Treat Immune Inhibitor in Classic Hemophilia

The life expectancy of a patient with classic hemophilia has been extended by the use of Factor VIII concentrates. However, development of an inhibitor to Factor VIII makes the standard form of treatment ineffective during bleeding episodes. Strauss (61) estimated that up to 21% of Type A hemophiliacs develop an antibody to Factor VIII, while other researchers (62) have reported the appearance of an inhibitor in approximately 7% of hemophiliacs. The inhibitors in hemophilia A are IgG antibodies, mainly monoclonal with kappa light chains. They are species specific and act specifically against Factor VIII procoagulant activity. It is unknown why

some hemophiliacs develop an antibody and others do not. It may be related to higher exposure to Factor VIII or merely reflect the tendency of some patients to produce antibodies more readily.

Management of patients with inhibitors during bleeding episodes has involved plasmapheresis (63) (64), immunosuppressive therapy (65) (66) and administration of bovine and porcine Factor VIII (67). Plasmapheresis has been ineffective in completely removing or diluting the circulating antibody and provides only short term improvement of the bleeding episode. There is conflicting data as to the suppression of antibody titers of immunosuppressive drugs such as cyclophosphamide, chlorambucil and 6-mercaptopurine. Bovine and porcine Factor VIII have been used with limited success. The higher level of Factor VIII in animal species enables use of a smaller therapeutic volume. However, allergic reactions to the animal concentrates are serious disadvantages. Another therapeutic approach has been neutralization of the inhibitor by continuous infusion of large amounts of Factor VIII. This has not been uniformly effective.

For the treatment of hemophiliacs with an inhibitor, the most dramatic response has been the use of prothrombin complex concentrates. Fekete et al (68) first reported a unique therapeutic approach using an "activated prothrombin complex" product (produced by Hyland Laboratories, Chicago, Illinois). From 1972 to 1976 there were five other reports of the

efficacious use of Factor IX concentrates for hemophiliacs with an immune inhibitor (69), (70), (71), (72), (73). All investigators reported control of the hemorrhagic episode after infusion of the concentrate. Both activated Factor IX concentrates (auto Factor IX) which contain trace amounts of Factors Xa, IXa and other coagulants, and standard concentrates such as Proplex (Hyland Laboratories) were used. More recent studies (74), (75), (76), (77) have used a new concentrate, FEIBA (Factor Eight Inhibitor By-Passing Activity). This product does not contain any Factor VII. FEIBA concentrates (Fraction R made by Immuno AG, Vienna, Austria) have been successful in controlling hemorrhagic complications of the disorder and are widely used in Europe.

The non-activated products which are currently available in the United States are Konyne and Proplex. These, however, for reasons unknown, have been less effective since 1976 for the management of the bleeding episode. The activated product available in the United States in current use is Autoplex (Hyland Laboratories).

Complications of Therapy With Prothrombin Concentrates

Potential complications of post-infusion prothrombin complex concentrates have been recorded. The reported incidence of viral hepatitis is high after infusion of Factor IX concentrates, but varies from country to country (78), (79), (80). The risk of hepatitis B virus transmission increased

when Factor IX concentrates were manufactured from paid commercial donors as opposed to volunteer donors. It has been recommended (81) that even though present screening techniques are not 100% effective in detecting blood or plasma contaminated with hepatitis B virus, all manufacturers should perform third generation testing. These tests include radioimmunoassay, reversed passive hemagglutination and the agglutination flocculation test.

Another complication has been an increase in the Factor VIII inhibitor concentration after infusion (82), (83), (84).

A third concern regarding the use of the concentrates is their thrombogenic properties. Menache (85) first noted the potential thrombogenicity of these concentrates. The greatest number of thrombotic episodes has occurred after infusion of concentrates into patients with liver disease. This may be due to decreased clearance by the damaged liver of activated factors present in the concentrates and decreased levels of antithrombin III. Fatal thromboembolism has been reported (86). Consequently, several investigators have strongly discouraged the use of prothrombin complex products in patients with liver disease.

The incidence of thrombotic complications resulting from the use of prothrombin concentrates has been low. Studies have shown (87) that several commercial preparations do contain substantial amounts of potentially thrombogenic enzymes and are able to produce thrombi in animal models. Thromboembolic

complications have been recorded with non-activated products as well as FEIBA (Immuno). Some researchers (88) (89) have suggested the addition of heparin to the reconstituted material to reduce thrombogenicity.

The mechanism of the hypercoagulable state after infusion of the complex is not completely understood. It may be due to the presence of Factor Xa or IXa, or the concentrates may be thrombogenic under certain conditions *in vivo* (90). It may be important to infuse a concentrate immediately after reconstitution since Elodi (91) reported generation of Factor IXa during incubation of the concentrate at room temperature for 20 minutes.

In vitro screening techniques have been proposed to test new batches of Factor IX concentrates for thrombogenicity. Blatt *et al* (92) suggested conduction a "pre-infusion stability check". This involves the addition of plasma to reconstituted concentrate at room temperature. If no clot forms after five minutes, the concentrate is assumed safe for administration. Kingdon *et al* (93) introduced a test in which Factor IX concentrates were added to a nonactivated partial thromboplastin time system (NAPTT). Shorter reaction times indicated possible thrombogenicity. Cash *et al* (94) found major discrepancies between the NAPTT and the thromboplastin generation test (TGt_{50}). The TGt_{50} represents the incubation time required to give a fibrinogen clotting time of 50 seconds. Products which had a short TGt_{50} were those in which thrombin

generation occurred more readily. Cash et al (95) concluded that the TGt_{50} correlated more closely than the NAPTT with in vivo systems, although it could not be recommended as the only monitor for thrombogenicity testing.

Mechanisms Responsible for By-Passing Factor VIII Inhibitor

There are many theories attempting to explain the mechanism of the coagulant effect of the prothrombin concentrates in the hemophiliac with an inhibitor. Kurczynski (96) suggested the presence of Factor IXa, Xa, or the mass effect of various factors on coagulation are responsible for clot promoting activity. Elsinger (97) reported that by-passing of the inhibitor is due to presence of Factors VII, IX, and X. Elsinger gave evidence that the presence of Factors VII, IX, and X are responsible for by-passing the Factor X activity although Factor V is not by-passed. In his experiments with the reaction of fraction FEIBA (active portion of preparation) and soybean trypsin inhibitor, he gave evidence to support the theory that the active component is not Factor Xa itself.

Vermylen (98) proposed the enhancement of Factor X activator activity of platelet, independent of the intrinsic or extrinsic cycle, is the by-pass mechanism. He found that platelets, prepared from plasma pre-incubated with fraction FEIBA, have increased coagulant activity. This can be demonstrated by decreasing the latent period of aggregation. This theory proved correct in patients who were infused with

FEIBA (Immuno). It was discovered that three hours post infusion there was a dramatic increase in the coagulant activity of the patients' washed platelets.

Tishkoff (99) in 1976 postulated that the active protease in the concentrates may be a binary complex of Factor X and thrombin based on molecular size measurements. Factor V is also a necessary component of this reaction. At that time, he suggested that this mechanism was an alternate extrinsic pathway for the generation of prothrombokinase. Factor X and thrombin, when reacted in a modified APTT, have the ability to by-pass the Factor VIII inhibitor substrate plasma to shorten the APTT.

CURRENT STATUS OF PROTHROMBIN COMPLEX CONCENTRATES IN MANAGEMENT OF BLEEDING EPISODES IN INHIBITOR PATIENTS

The commercially available products used today in the United States are Konyne^{R} and Proplex^{R}. AutoProplex (HYLAND) is used with a limited basis. Several problems remain concerning the clinical use of the prothrombin concentrates. Because the exact mechanism is unknown, there is no standardization of the commercial concentrates. This accounts for a wide degree of variation in the products. There is still the potential for thrombogenic complications as well as anamnestic response in some patients. Finally, there have been in the past few years conflicting reports as to the efficacy of the concentrates in managing a bleeding episode.

Materials and Methods

Chromatographic Materials

Sephadex G-100, G-200, BioGel A-15M, DEAE-A50, and all column chromatographic equipment were purchased from Pharmacia Five Chemicals, Piscataway, New Jersey. Whatman DEAE Cellulose was obtained from H. Reeve Angel and Co., Clifton, New Jersey. Dowex 50X8 (20-50 mesh, Na form) from J.T. Baker Chemical Co., Philipsburg, New Jersey. Hydroxyapatite, Bio-Rex 70 (110-200 mesh), and Chelex resin (100-200 mesh, Na form) were purchased from Bio Rad Laboratories, Richmond, California.

Equipment

Major equipment used included a Beckman Du Spectrophotometer, Fullerton, California, with Gilford light source and cuvette position, Oberlin, Ohio; Radiometer pH meter, Copenhagen, Denmark; LKB fraction collector, Rockville, Maryland; Amicon concentrating ultrafilter system, Lexington, Massachusetts; Bio Rad ultrafilter beaker; and Sorvall centrifuge, DuPont Instruments, Newtown, Connecticut.

Coagulation Reagents.

Bovine plasma deficient in Factors VII and X, crude Rusell's viper venom in cephalin, human Factor V and heparin, grade 1, sodium salt from porcine intestinal mucosa were all obtained from Sigma Chemical Co., St. Louis, Missouri. Activated thromboplastin and activated cephaloplastin (ACTIN) were purchased from Dade, Miami, Florida. Human fibrinogen, grade L, was obtained from ABKABI, Stockholm, Sweden. The fibrinogen was dialyzed to reverse excess citrate which interfered with the two-stage prothrombin assay. The fibrinogen (1.5g dissolved in 66 ml of distilled H_2O) was dialyzed overnight at 4C against 4 liters of buffer consisting of 35.35g NaCl, 2.87g sodium citrate, 17.74g sodium acetate. It was subsequently frozen at -80C in 10 ml aliquots. Factor VIII Inhibitor plasma was donated by Dr. J. Penner, then of the University of Michigan, Ann Arbor, Michigan.

Chemicals

Acrylamide, methylenebisacryalamide, N,N,N',N' - tetramethylethylene - diamine (TEMED), ammonium persulfate, sodium dodecyl sulfate, dithioerythritol, and all electrophoretic equipment was purchased from Bio Rad Laboratories. Ethanolamine, benzamidine-hydrochloride (HCl) was obtained from Eastman Organic Chemicals, Rochester, New York. Blue dextran 2000 was obtained from Pharmacia Fine

Chemicals. Cyanogen bromide and polybrene were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Australian Taipan venom and Soybean trypsin inhibitor was purchased from Sigma Chemical Company. Kabi chromogenic substrates S2160 and S2238 were purchased through Ortho Diagnostics, Raritan, New Jersey. All other chemicals were reagent grade and all solutions prepared with distilled, deionized water.

Protein Determinations

Protein was monitored by absorbance readings at 280 nm. Protein concentrations were then calculated according to the formula: (OD at 280 nm)(0.795) + 0.006 = mg protein per ml.

Conductivity Determinations

A radiometer type CDM 2e conductivity meter was utilized to measure conductivity. The readings of unknown samples were compared to standard curves that were constructed using buffers of known ionic strengths.

Coagulation Assays

Prothrombin and thrombin were assayed by modifying the 2 stage prothrombin procedure of Ware and Seegers (100) by replacing bovine fibrinogen with human fibrinogen. The results are expressed in Iowa units. One unit of prothrombin yields one unit of thrombin. Normal plasma values assayed 250 units of prothrombin per ml.

Factor X and Xa were assayed according to the method of Bachman (101). Factor X was assayed using bovine plasma deficient in Factors VII-X and Russell's viper venom. One unit of Factor X activity is equivalent to the activity in one ml of normal human plasma. Factor Xa was assayed by substituting rabbit brain cephalin for Russell's viper venom and comparing the clotting times obtained to the Factor X standard curve.

Factor VIII was assayed according to a modified one stage Quick (102) procedure using human Factor VIII deficient plasma donated by Dr. J. Penner.

Factor IX was assayed according to the method of Proctor and Rappaport (103).

Standard curves were prepared for all clotting assays utilizing a fresh plasma pool from male donors. The anticoagulant was 3.8% sodium citrate.

Preparation of dialysis tubing

Dialysis tubing was treated according to Methods of Enzymology (104).

Antithrombin III

The partially purified human antithrombin III was obtained from the National Red Cross Research Laboratory, Bethesda, Maryland. It was further purified on a Sephadex G-100 column before using it in the assay procedures.

Human Plasma

Plasma was obtained from the Great Lakes Regional Red Cross Services, Lansing, Michigan. Three inhibitors were added to the fresh plasma as follows: 0.157g/liter Benzamidine - HCl; 50 mg/liter polybrene; and 20 mg/liter of soybean trypsin inhibitor. The plasma was then centrifuged for 30 minutes, at 10,000 rpms at 4C. The supernatant was collected in plastic transfer bags and frozen at -80C overnight. It was then slowly thawed at 4C and centrifuged at 8,000 rpm for 15 minutes and the supernatant (cryo-precipitate poor plasma) finally stored in plastic containers at -80C.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate gels (SDS) were prepared according to the method of Fairbanks (105).

Molecular weight markers purchased from Schwartzman, Orangebury, New York, were run in the same manner as the protein solution. They consisted of phosphorylase A, albumin, gamma globulin, and cytochrome c. The mobility of the known proteins was plotted against the log of their molecular weights. Unknowns were compared to the standard curve to obtain an apparent molecular weight.

Factor VIII Inhibitor Bypass Activity (FEIBA) Assay

An assay was developed based on the correction of the abnormal APTT of Factor VIII inhibitor plasma after a 1-minute and 40-minute incubation time. The inhibitor assay consisted of adding 0.1 ml of Factor VIII Inhibitor plasma to 0.1 ml of highly purified Factor X, Factor II, Factor IIa or a combination of Factor X and IIa and allowing the proteins to incubate at 37C for 1 minute and 40 minutes. At the appropriate times, 0.1 ml of activated cephalaplastin (ACTIN) was added for an additional 2 minutes incubation. After allowing the lipid to interact, 0.1 ml of 0.02M calcium chloride was added, a stop watch started and the clotting time recorded. Dilutions of proteins in the assay were made with sodium barbital buffered saline, pH 7.4. (VBIS).

Chromogenic Substrate Assay

Direct absorbance assays were utilized and the reaction was as follows:

0.1 ml purified Factor X (0.4 clotting units of activity in the assay)

0.1 ml Tris Imidizole buffer (pH 7.4) or

0.1 ml purified thrombin (0.015 clotting units in assay)

0.1 ml activated Cephaloplastin (SIGMA)

0.1 ml 0.02M calcium chloride

0.1 ml purified human Factor V (SIGMA)

The above reactants were pipetted into a pre-warmed cuvette (37C) and allowed to incubate for various times. At the appropriate time, 0.1 ml of purified human antithrombin III (22 ug in assay) and 0.1 ml sodium heparin (UPJOHN) were added for an additional 15 minute incubation. At this point, 0.1 ml S2238 (Ortho) diluted to 0.1 mM in the assay was added and the recorder turned on. An endogenous rate was established during a 2-minute period, which was followed by the addition of 0.1 ml highly purified human prothrombin (250 units of activity in the assay). The rate of para-nitroaniline production was followed at 405 nm.

Calculations for converting the spectrophotometric data into linear plots were based on Kosow's kinetic analysis (106) in which the following formula was used:

$$X - V_o t + 1/2\ at^2$$

where V_o equals the velocity at zero time and is the endogenous rate of hydrolysis, t equals time, a equals acceleration, and X equals absorbancy. When $V_o t$ is subtracted from the absorbancy (X) this value will be X corrected. The X corrected value is then plotted against $(time)^2$ to obtain a straight line. Acceleration, a, is proportional to the velocity of the reaction.

Preparation of Blue Dextran Resin

Preparation of this affinity column was performed according to the procedure of Vician and Tishkoff (107).

Preparation of Sephadex G-200

Sephadex G-200 was prepared according to the manufacturers' recommendations.

Preparation of DEAE-52

The anion exchange resin was prepared according to Methods in Enzymology (108).

Preparation of Heparin Sepharose

Preparation of this chromatographic material began with 30 ml of washed Bio Gel A-15M mixed with 30 ml of 2M sodium carbonate. With rapid stirring, 3 ml (0.05 volumes) of cyanogen bromide - acetonitrile solution was added and stirred vigorously for 1 to 2 minutes. The cyanogen bromide - acetonitrile solution was prepared by adding 50 ml of redistilled acetonitrile to 100g of cyanogen bromide. The slurry was then washed with 5-10 volumes of 0.2M sodium bicarbonate buffer, pH 9.5 and distilled water. The washing was done in less than 90 seconds. The slurry, plus one volume of a 2M sodium bicarbonate, was placed in a plastic bottle with 8-10 mg/ml of heparin.

The activated agarose - heparin mixture was stirred constantly for 18-20 hours at 4C. Unreacted groups were treated by adding 1 molar glycine and stirring for an additional 3 hours at room temperature. The coupled agarose was subsequently washed using sequentially 20 volumes each of 0.01M sodium bicarbonate/0.5M sodium chloride (pH 10.0), 0.1M sodium acetate/0.5M sodium chloride (pH 4.0), and 2M Urea/0.5M sodium chloride. The equilibrating buffer contained 5mM potassium chloride, 3mM calcium chloride, 10mM sodium acetate and 10mM Triethanolamine - HCl (pH 6.4).

Preparation of Hydroxyapatite Column

Hydroxyapatite (BIO RAD) was hydrated with 0.2M phosphate buffer, pH 6.8. The resin was defined and degassed and then poured all at once into a jacketed column, 2.5cm x 45cm. The column was equilibrated with 0.02M phosphate buffer (pH 6.8).

Preparation of Thrombin Sepharose

Thrombin Sepharose was prepared according to Rosenberg, et al (109) with modifications. Fourteen ml of packed Sepharose 4B was washed with distilled H_2O and then added to a beaker with 14.0 ml distilled H_2O, stir bar, pH electrode and thermometer. Finely ground cyanogen bromide (CnBr) was added all at once and the coupling reaction began. The temperature was kept between 18C-20C and the pH at 11.0.

Sodium hydroxide, 4M, was used to adjust the pH. The reaction proceeded for approximately 12 minutes at which time excess ice was added to the beaker and the gel washed with 500 ml of cold 0.1M $NaHCO_3$ (pH 8.5) and 14.5 ml of thrombin in 0.1M $NaHCO_3$ (pH 8.5). The washing step was completed in less than 120 seconds. The slurry was gently stirred overnight. One molar ethanolamine - HCl, pH 8.0, was added and stirred with the gel for 2 hours, after which the thrombin sepharose was washed with distilled H_2O. The gel was then stirred for 30 minutes in 0.1M carbonate buffer/1.0M NaCl, pH 8.90. The slurry was filtered and washed four more times with the carbonate buffer and stored at 4C.

Preparation of Thrombin

Prothrombin was activated to thrombin according to the method Lanchantin, et al (110) with minor modifications. Australian Taipan venom (oxyranus scutellatus scutellatus) was partially purified on a DE52 column to remove nonspecific proteolytic enzymes. Approximately 3 mg/ml of Australian Taipan venom was placed on a 1 x 10 cm column and run at 20 ml/hour, 6 minutes/tube. The protein was eluted with a buffer gradient of 0.02M TRIS pH 7.2, at 25C and 0.02M TRIS/1.0M sodium chloride. The venom was then eluted from the column at a conductivity reading of approximately 4.5 mmOHMs. The purified venom was reacted with purified

prothrombin in a ratio of 0.7 mg venom to 15 mg of Factor II. The reaction took place at 28C for 30 minutes with constant agitation.

The reaction mixture was then placed on a Bio Rex 70 (100-200 mesh) column (1.6 x 70cm) which had been equilibrated with two bed volumes of 0.1M acetate buffer (pH 7.0). The Bio Rex was swollen directly in the buffer without preliminary washing procedures. It was poured in three sections to allow for better uniformity. The column was run at 8 minutes/tube, 12 ml/hour. Protein was eluted with a buffer gradient consisting of 120 ml of 0.1M acetate buffer pH 7.0 and 200 ml of 0.1M acetate buffer/1.0M sodium chloride. Conductivity measurements and thrombin clotting assays were performed on the protein peaks.

Preparation of AT-III Sepharose Column

The Antithrombin III (AT-III) obtained from the American Red Cross, Bethesda, Maryland, was prepared from fresh frozen plasma by the method of Wickerhouse and Williams (114). A Sephadex G-100 column was packed and 20 ml of the reconstituted AT-III was placed on the column. Imidizole buffered saline, pH 7.35, was used to elute the protein. Protein was monitored at an absorbance of 280 nm and AT-III assayed in terms of its capacity to inactivate thrombin during an incubation time. The AT-III was then dialyzed against sodium carbonate buffer, pH 8.5, for 7

hours. It was then attached to cyanogen bromide activated Sepharose 4B.

Purification of Factor X (Vician-Tishkoff's method)

One liter of cryo-precipitate poor plasma plus inhibitors (benzamidine - HCl, polybrene, and soybean trypsin inhibitor) was thawed at 37C in a water bath. The thawed plasma was poured through gauze into a plastic container on ice. After thawing, the plasma was stirred at 4C for 60 minutes with the addition of 100 ml of cold 1M barium chloride. The plasma was then centrifuged at 3500 rpm for 30 minutes at 4C. The supernatant was discarded and the pellet resuspended in 500 ml of cold distilled water. Dowex (30g of 50X 8, 20-30 Na mesh) was added to the resuspended protein and stirred for 30 minutes, after which it was filtered through a coarse sintered glass funnel. It was washed 3 times with 15 ml each of cold 3.1% sodium citrate. The protein solution was once again stirred for 30 minutes in an ice bath with the addition of 56 ml of cold barium chloride. It was then centrifuged in three plastic centrifuge tubes for 30 minutes at 3500 rpm. The supernatant was discarded and each pellet resuspended with 80 ml of 0.85% sodium chloride, and mixed for two minutes only. The centrifuge tubes were spun once more at 3500 rpm for an additional 30 minutes. The supernatant was discarded and the precipitate resuspended with 120 ml of cold

distilled water. Dowex (30g) was once again added to the protein solution and stirred for 30 minutes followed by filtering and washing with a total of 45 ml of cold 3.1% sodium citrate. The protein solution was then dialyzed overnight (approximately 16 hours) at 4C against 7 liters of phosphate/NaCl buffer, pH 5.8. After dialysis, the solution was passed through a chelex column which had been pretreated by washing with H_2O, 0.5M acetate buffer, (pH 5.4), H_2O, and phosphate NaCl buffer (pH 5.8) and then concentrated in a Bio Rad hollow fiber beaker at 4C. The concentrated protein was centrifuged at 15,000 rpm in corex tubes and filtered through glass wool. After approximately 30 liters of plasma were processed this way, the last step involving concentration of the protein by the hollow fiber technique was changed. An ammonium sulfate precipitation step was substituted. After the chelex column, solid $(NH_4)_2SO_4$ was added to the protein solution to yield a 0-40% precipitation. The $(NH_4)_2SO_4$ was added slowly, at 4C, for 15 minutes with constant stirring. The pH was adjusted with 2M NaOH to approximately 6.2. The protein solution was then centrifuged in small plastic tubes at 16,000 rpm for 30 minutes at 4C. The supernatant was saved and the appropriate amount of $(NH_4)_2SO_4$ was added to yield a 40-75% precipitation. The $(NH_4)_2SO_4$ was once again slowly added at 4C, with constant stirring. Once all the solid was in the solution it was stirred for 30 minutes. The pH was again

adjusted to approximately 6.2. The protein solution was centrifuged at 16,000 rpm for 30 minutes. The supernatant was discarded and the pellets resuspended in about 5 ml of Sephadex phosphate/NaCl buffer plus inhibitors. The solution was dialyzed and then placed on the gel filtration column, Sephadex G-200.

The isolated prothrombin complex was first chromatographed on a Sephadex G-200 column. The fraction collector was run at a flow rate of 20ml per hour and fractions collected at intervals of 15 minutes per tube. Protein was eluted with phosphate/NaCl buffer (pH 5.8) plus inhibitors and monitored at an absorbance of 280 nm. Clotting assays were performed on the eluted protein. The pooled protein was concentrated and dialyzed against 0.1M acetate buffer (pH 6.0) overnight at 4C.

The second column utilized in the purification of Factor X was a Blue Dextran agarose affinity column prepared as previously described. The column was run at 20 ml/hour and 12 minutes/tube. Protein was eluted with a stepwise gradient of acetate buffer (pH 6.0) consisting of: 0.01M sodium acetate/acetic acid, and 0.4M sodium acetate acetic acid plus 0.2M sodium chloride. The column was regenerated with 300 ml of 0.1M sodium carbonate/1M NaCl (pH 8.8) and then reequilibrated with 0.01M sodium acetate buffer (pH 6.0).

The final column used in this purification process was an anion exchange column, DE52. The protein was eluted with an exponential buffer gradient using sodium phosphate, pH 6.1. The flow rate was 20 ml/hour at 4C.

Conductivity measurements and clotting assays were performed on the eluted protein. Factor X eluted from the final column was pooled, dialyzed against sodium barbital buffered saline, pH 7.4 and stored at -80C.

Purification of Factor X (Kosow's method)

Factor X was also purified according to Kosow's method (111) which utilized three types of chromatographic material: DEAE-A50, heparin agarose, and hydroxyapatite. The procedure began with thawing 2 liters of cryo-poor plasma plus inhibitors, collected as previously described. The plasma was stirred at 4C for one hour with 3.7g of preswollen DEAE-A50 which had been swollen and defined in the following buffer: 10mM triethanolamine - chloride, 0.75 mM potassium chloride, and 2mM EDTA. The protein was then poured into a Bel Art funnel and washed with the following buffer: 10mM triethanolamine - chloride, 0.2M potassium chloride, and 2mM EDTA. The protein solution and resin were then poured into a 2.5cm x 45 cm column and the gel allowed to settle. The column was run at 20 minutes/tube, 10 ml/tube and 30 ml/hour. Protein was eluted with a buffer gradient of 500 ml of starting buffer which consisted of:

10mM triethanolamine - Cl, 0.2M KCl, 2mM EDTA; and 500 ml of limit buffer consisting of: 10 mM triethanolamine - Cl, 0.5M KCl, 2mM EDTA. Factor X clotting assays were performed and tubes with the highest specific activity were pooled. Two $(NH_4)_2SO_4$ precipitations were done on the pooled protein. The first involved adding dry $(NH_4)_2SO_4$ to yield a 0-40% precipitation. The dry solid was added slowly at 4C and stirred for one hour. The pH was adjusted to 7.2. The protein was then centrifuged at 16,000 rpm for 30 minutes. The supernatant was saved and $(NH_4)_2SO_4$ added to yield a 40-75% precipitation. The solution was once again stirred, pH adjusted and centrifuged for 30 minutes at 16,000 rpm. The pellet was resuspended in starting buffer for the heparin agarose column which consisted of: 10mM triethanolamine - acetate, 5mM KCl, 3mM $CaCl_2$ (pH 6.4). The resuspended protein was dialyzed against the heparin agarose starting buffer overnight.

The protein was then applied to the heparin agarose affinity column with a flow rate of 30 ml/hour and 12 minutes/tube. The buffer gradient was applied after the first protein peak was eluted. The buffer gradient was 300 ml of starting buffer (listed above) and 500 ml of limit buffer which was like the starting buffer with the addition of 500 mM KCl. Conductivity measurements and Factor X, Xa clotting assays were performed on the eluted protein. Tubes with the highest specific activity were pooled and dialyzed

against starting buffer for the last column, the hydroxyapatite column.

The protein was applied with a flow rate of 30 ml/hour, 12 minutes/tube. The protein was eluted with 500 ml of 0.2M $KHPO_4$ and 500 ml of 0.4M $KHPO_4$ (pH 6.8) both prepared with degassed, deionized water. Conductivity measurements and clotting assays were performed. Tubes with the highest specific activity were pooled and concentrated with an Amicon system, membrane PM10. The concentrated protein was then dialyzed against sodium barbital buffered saline and stored at -80C.

Isoelectric Focusing

Isoelectric focusing was performed according to LKB Application Note 250: Analytical Electrofocusing in Thin Layers of Polyacrylamide Gel.

The standards run with the unknown proteins were catalase, ovalbumin, ribonuclease and cytochrome c mixed together for a final protein concentration of 0.03 mg/ml.

Reduction and Alkylation of Factor X

The Factor X was reduced and alkylated according to Summaria (112) with minor modifications. The procedure was first done with gamma globulin to ensure proper reduction reactions. The reducing reaction mixture consisted of 5.2 ml of globulin (approximately 0.5 mg/ml), plus 1.0 ml of DTE

(0.077g DTE in 1.0 ml of barbital buffered saline, pH 6.0). The final pH of this reaction mixture was 6.95 at 23C. The reaction took place for 20 minutes at 23C. The alkylating step involved adding 1.24 ml of iodoacetate (0.1M in combination with gamma globulin and DTE) and adjusting the pH to 7.0 with 1M NaOH. The reaction was complete when there was a decrease in pH change. The reaction mixture was then dialyzed overnight against sodium barbital buffered saline (VBIS), pH 7.4, at 4C.

Factor X was reduced and alkylated in a similar manner. Factor X, 2.8 mg/ml, was reacted with 0.48 ml DTE (77 mg/ml) at 23C for 20 minutes. The alkylating step involved adding 0.66 ml of 0.5M iodoacetate and adjusting the pH to 6.0 with 0.2M NaOH. The reaction mixture was likewise dialyzed overnight at 4C against VBIS.

Activation of Factor X

Factor X was activated (113) in a reaction with purified Russell's viper venom and 0.2M $CaCl_2$. The weight ratio of X to protease was 14.3:1. The reaction time was approximately 5 minutes. The activated Factor X was then placed on a Sephadex G-100 column and the protein was eluted with sodium phosphate/sodium chloride buffer, pH 6.1.

Antisera Production

Factor X used for injection was purified by Kosow's method. Protein concentration was 0.32 mg/ml. On ml was diluted with 2 ml of phosphate buffered saline, pH 6.0. Therefore, final Factor X concentration was 107 mg/ml. The protein was then diluted 1:1 with Freunds Complete Adjuvant (Grand Island Biological Company). Two white male rabbits (2kg) were each injected subcutaneously along the back with 0.5 ml of this mixture. The second injection was given six weeks later. The rabbits were bled on days 12 and 34 following the second injection. They received boosters one month after the second bleeding.

Results

Purification of Human Factor X - Vician and Tishkoff's Method

Factor X was successfully purified 2,000 fold by Vician and Tishkoff's method (116).

The prothrombin complex extracted from cryo-precipitate poor plasma or from a commercial concentrate was first chromatographed on a gel filtration column, Sephadex G-200. The prothrombin complex was conveniently eluted in the second peak. Prothrombin complex chromatographic patterns off the G-200 column are illustrated in Figure 5 and Figure 6. Samples containing Factor X activity, and some Factor II and VII activity, were then pooled, concentrated and dialyzed against starting buffer for the second column, Blue Dextran agarose (BDA).

The BDA column exhibited a unique affinity for separating out the Factor X from the remainder of the prothrombin complex. The typical procoagulant profile in which Factor X is conveniently eluted in the first or second peak, while Factors II, VII and IX are eluted at higher ionic strengths is illustrated in Figure 7. Approximately 95 liters of cryo-precipitate poor plasma were processed by this method. Some of the fractionated patterns did show

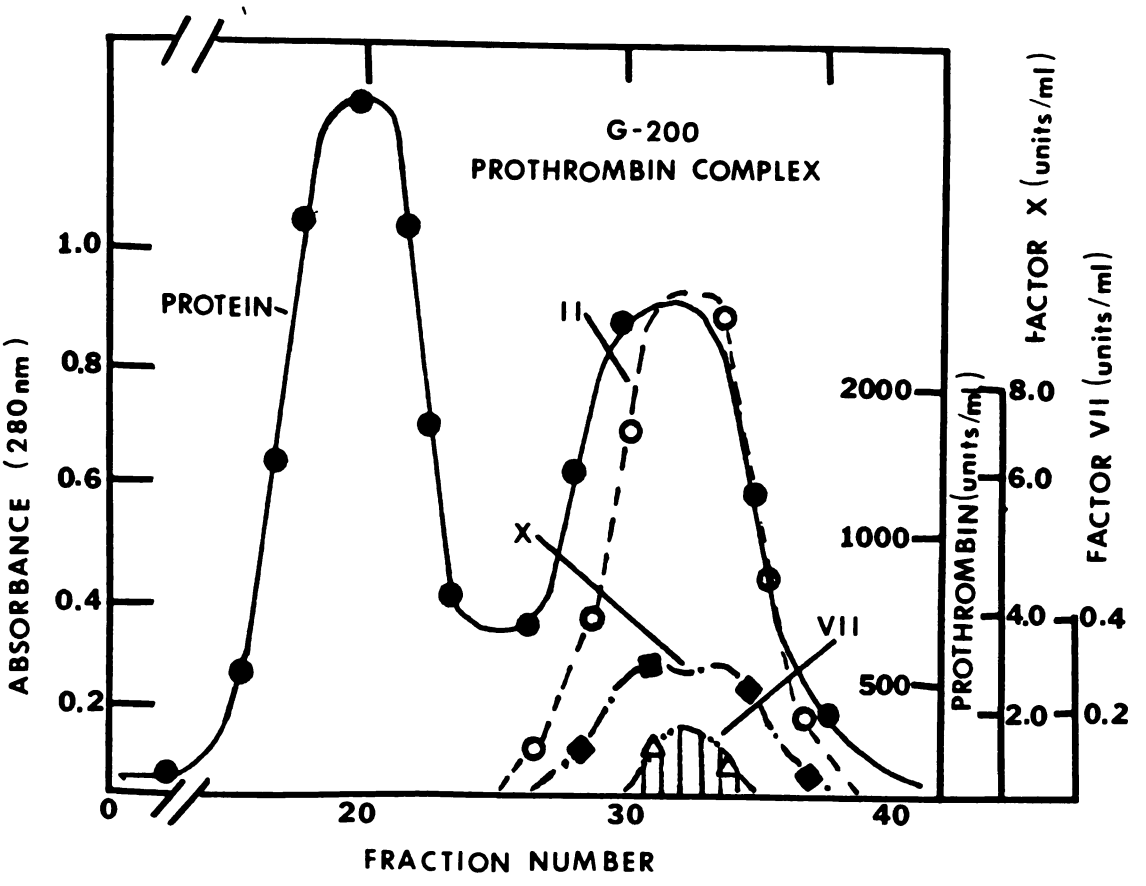

Figure 5. Procoagulant profile of prothrombin complex eluted from Sephadex G-200

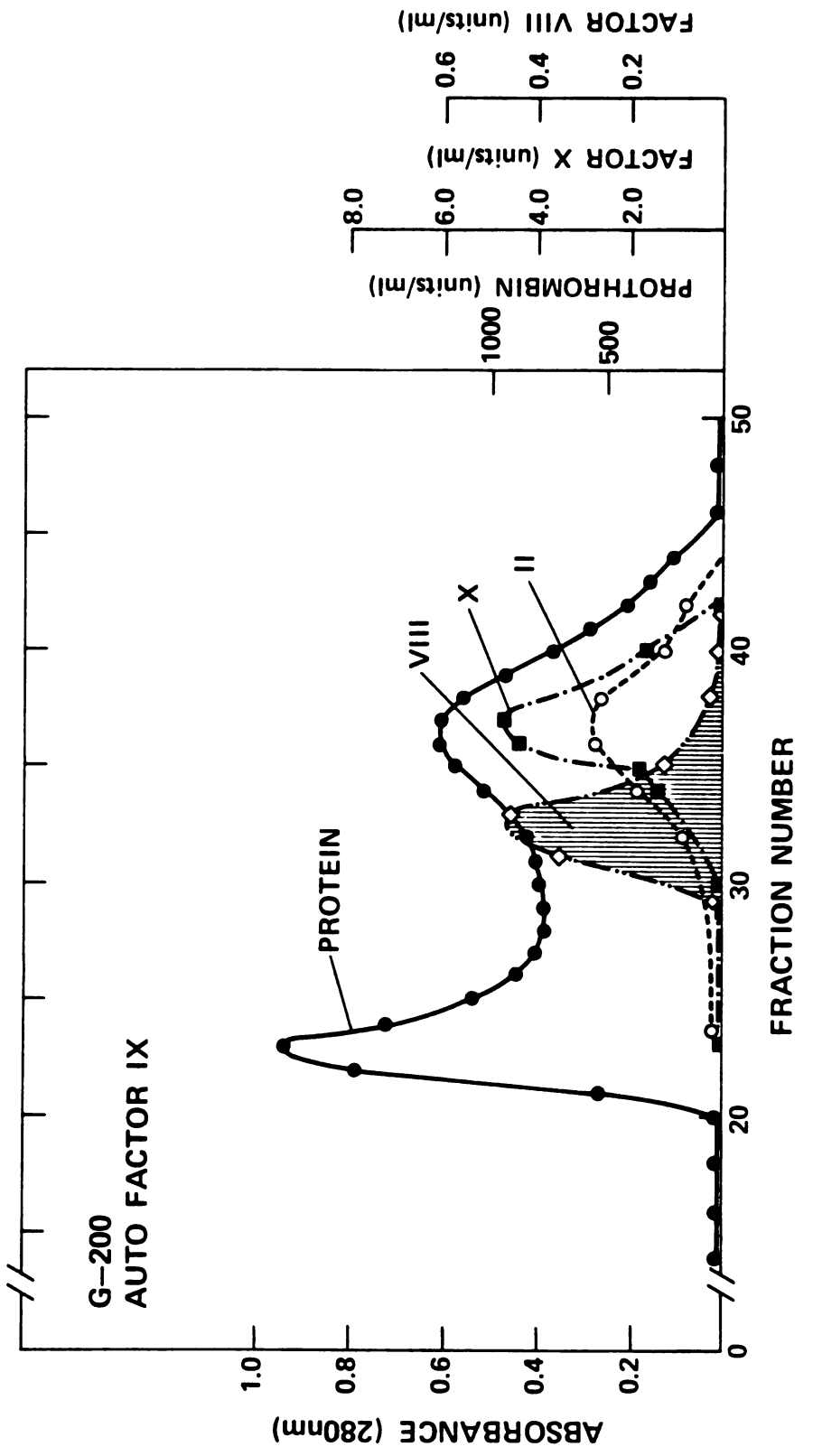

Figure 6. Procoagulant profile of Auto Factor IX (HYLAND) chromatographed on Sephadex G-200

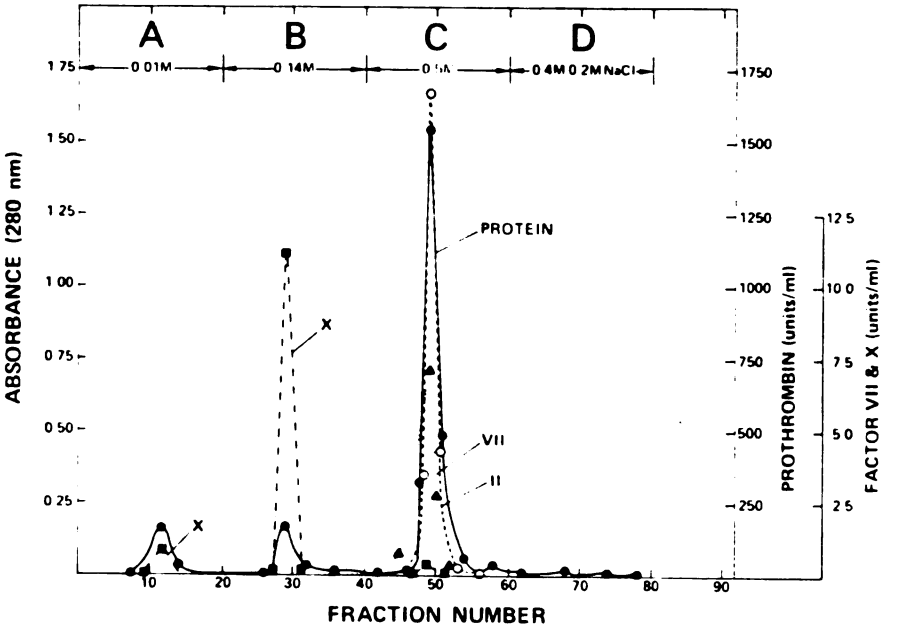

Figure 7. Pooled prothrombin complex chromatographed on Blue Dextran resin

patterns where the Factor X was eluted with only the first or second buffer. Several times, Factor VII was separated from Factor II. In these cases, Factor VII was eluted prior to the prothrombin complex. This occurred more frequently when the prothrombin complex was absorbed from cryo-precipitate poor plasma and subsequently precipitated with ammonium sulfate. Clotting assays were performed and samples with the highest amount of Factor X activity were pooled, dialyzed against VBIS, pH 7.4, and stored at -80C. Several preparations of Factor X were then thawed, pooled, and dialyzed against the starting sodium phosphate buffer for the DE52 column.

Approximately 5.0 to 10.0 mg of protein was placed on the DE52 ion exchange column. Conductivity readings and clotting assays were done. A typical elution pattern is illustrated in Figure 8. Factor X fractions containing the highest specific activity were immediately dialyzed against VBIS (pH 7.4) and stored at -80C.

Data in Table 2 summarizes results from several preparations and is a representative sampling of the Factor X purification.

The purification process listed in Table 2 involved the hollow fiber concentrating step and not the ammonium sulfate precipitation. Factor X in the final product, is devoid of Factor II, VII and IX clotting activity. Factor Xa activity

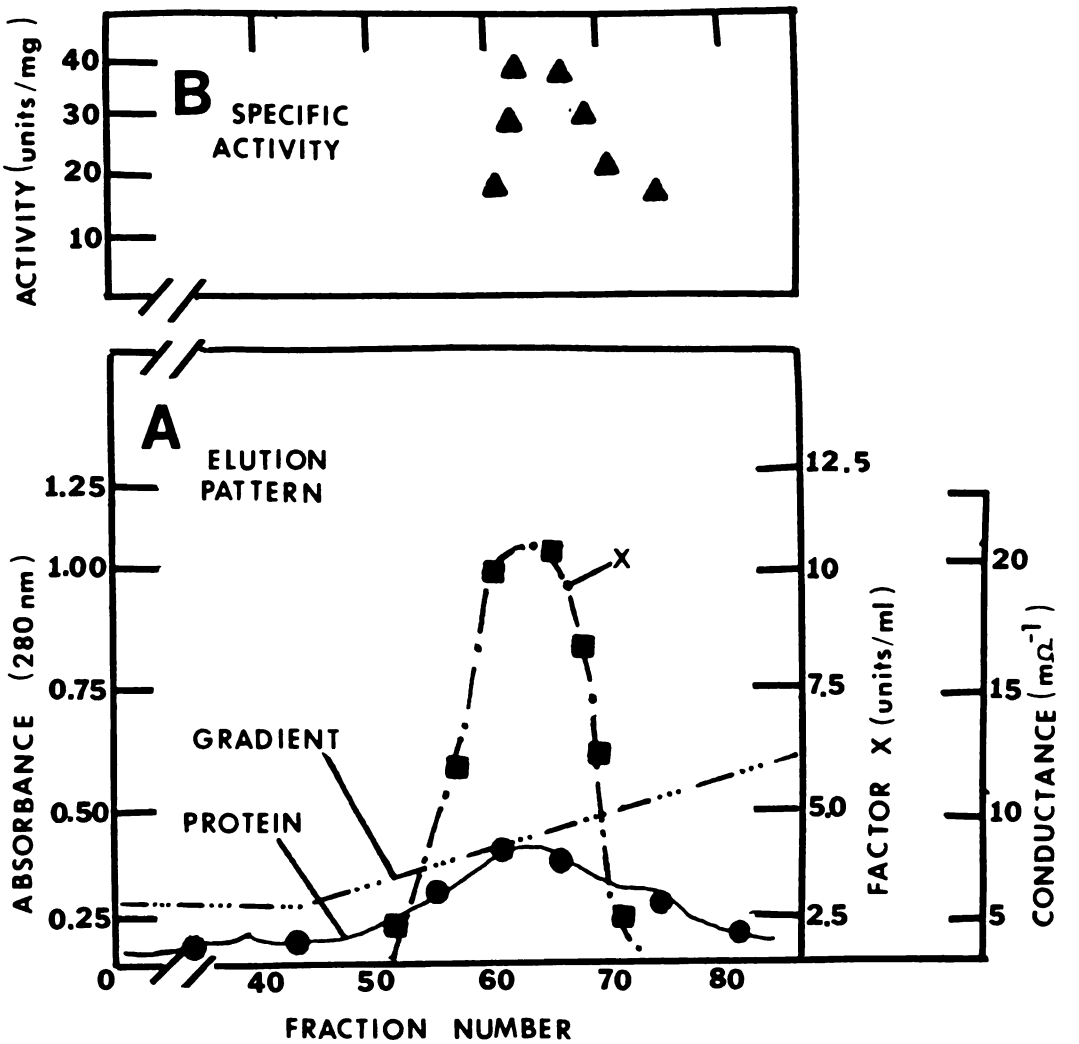

Figure 8. Chromatography pattern of Factor X protein off ion exchange resin (DE52)

TABLE 2 - Human Factor X Purification (Vician and Tishkoff): A Summary of Several Preparations

Material	Volume (ml)	Protein (mg/ml)	Total protein (mg/ml)	Protein recovery (%)	Factor X u/ml	Factor X (units)	Factor X recovery (%)	Spec. act. (u/mg prot)	Purification fold
Human Cryo-poor Plasma	95.0	42.0	37,595	--	.69	599.4	--	0.014	1.0
Chelex Resin Eluant	16.4	3.35	575.8	100	2.18	343.3	6.2	.680	46.7
After Concen-trating in Hollow Fiber Beaker	33.5	12.63	422.1	76.7	13.0	468.0	--	1.105	85.0
After Centri-fuging	29.0	8.06	232.6	40.3	12.25	3545	77.6	1.575	130.9
Sephadex G-200 Pool	202	0.38	66.1	11.7	1.05	236.3	41.4	3.08	220.6
After Concen-trating off G-200	28	2.415	68.0	11.8	6.30	177.5	38.5	2.62	218.15
Blue Dextran Affinity Pool	13	0.13	-	-	6.00	78.0	8.8	47.0	2502.0
DEAE-Cellulose (DE52)	-	0.10	-	-	3.60	-	-	-	1951.0

is less than 0.01 u/ml. Specific activity is approximately 30-50 units/mg protein.

Factor X appeared homogeneous on polyacrylamide gels performed according to the method of Fairbanks (117) with intact disulfide bridges; a single band is seen for both molecular species of X (the two peaks off the BDA column). Following disulfide reduction, Peak 1 (off BDA), Factor X evidenced a 2-chain species, while the second peak is predominantly a single chain species of Factor X with a molecular weight of 85,000 daltons (Figure 9).

Purification of Human Factor X - Kosow's Method

Factor X purified by Kosow's method (118) yielded a product with a specific activity of approximately 100 units/mg of protein.

The chromatographic pattern of Factor X off the DEAE-A50 is illustrated in Figure 10. Fractions containing the highest specific activity of Factor X were pooled and two ammonium sulfate precipitations were performed.

The protein elution pattern of Factor X off the heparin agarose column is shown in Figure 11. Peak Factor X activity fractions were pooled, dialyzed against 0.2M potassium phosphate buffer for 2 hours, and concentrated with an Amicon system, membrane PM10.

The concentrated protein was applied to the hydroxyapatite (HT) column and protein eluted with a linear

Figure 9. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified Factor X (Vician-Tishkoff's method)

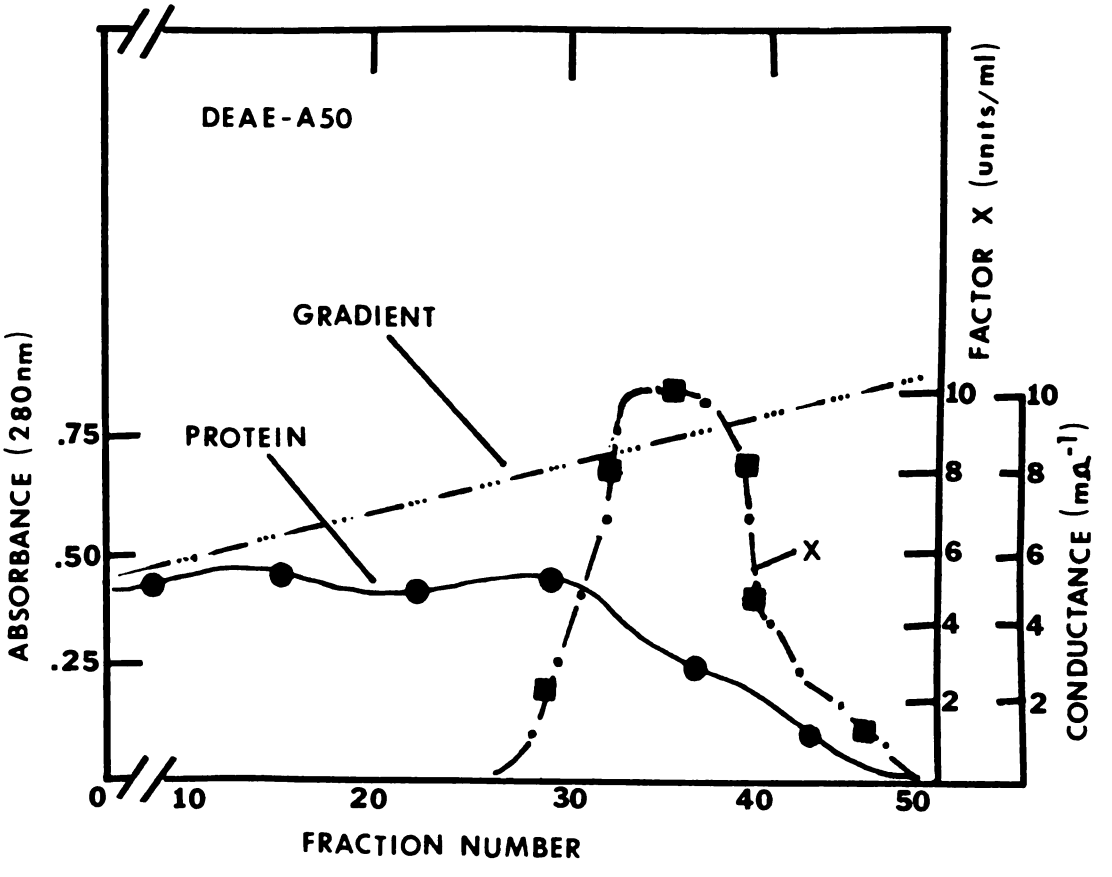

Figure 10. Elution pattern of prothrombin complex from DEAE-A50

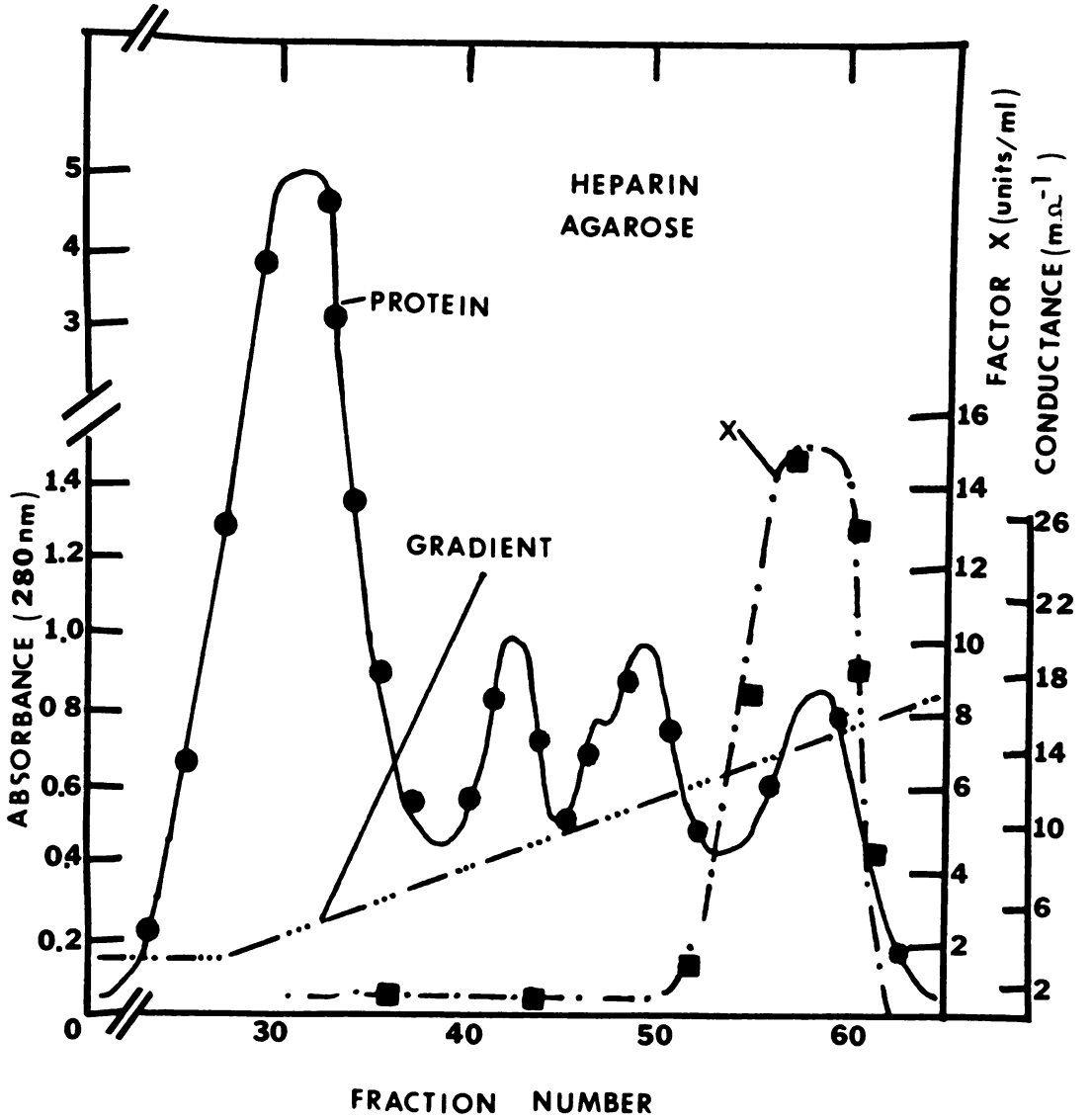

Figure 11. Protein profile of Factor X from heparin agarose column

gradient of 0.2M-0.4M potassium phosphate, pH 6.80. A typical profile of Factor X eluted from the HT column is presented in Figure 12. The final product was devoid of Factor II, VII and IX activity. Factor Xa activity was less than 0.01 units/ml. The specific activity of the purified Factor X was approximately 100 units/mg of protein. Sodium dodecyl sulfate gels showed Factor X to have intact disulfide bridges and a molecular weight of approximately 70,000 daltons. There appeared to be a high molecular weight contaminant present on most preparations. These results are consistent with Kosow's observations (Figure 13).

Activation of Prothrombin to Thrombin

The elution pattern of Australian Taipan venom off an ion exchange column is illustrated in Figure 14. This procedure was performed several times, with similar elution patterns being obtained. The venom was eluted when conductivity readings were equal to the standard curve readings for 0.02M TRIS/0.1M NaCl. As shown in Figure 14 the second peak was the partially purified venom.

The activation took place as described in materials and methods, the ratio of venom to prothrombin by weight was 0.7 mg:15 mg, respectively. Data presented in Figure 15 illustrate the Bio Rex elution pattern. Thrombin assays, conductivity readings and absorbance measurements were done

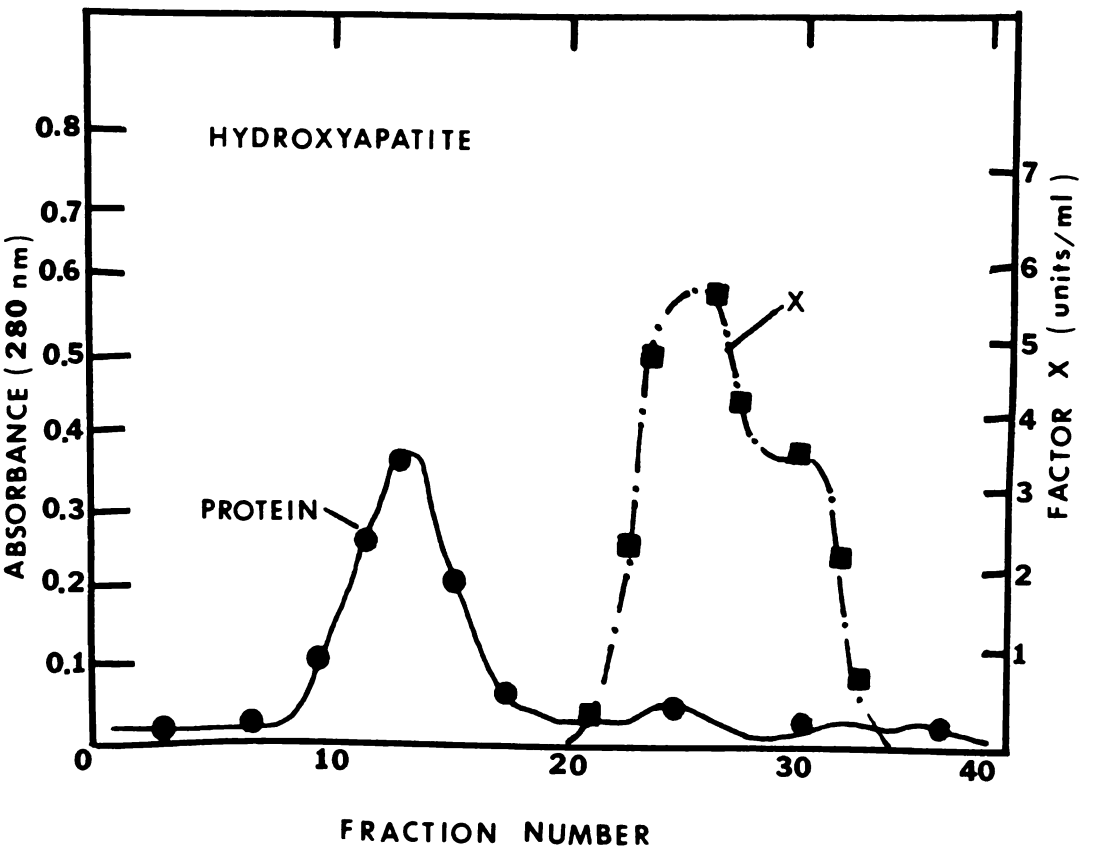

Figure 12. Factor X pattern off of Hydroxyapatite column

Figure 13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified Factor X (Kosow's method)

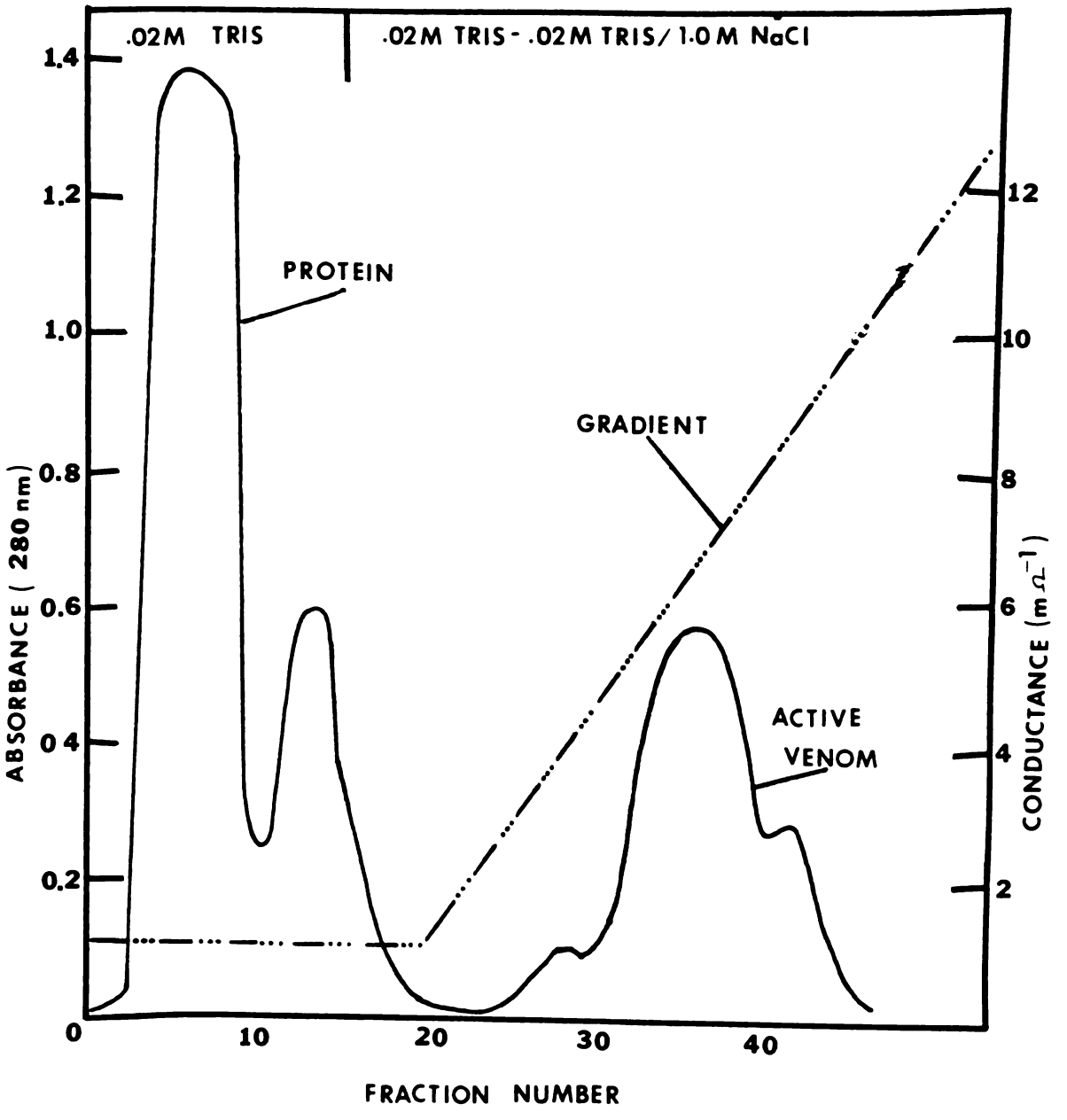

Figure 14. Australian Taipan Venom chromatographed on DE52

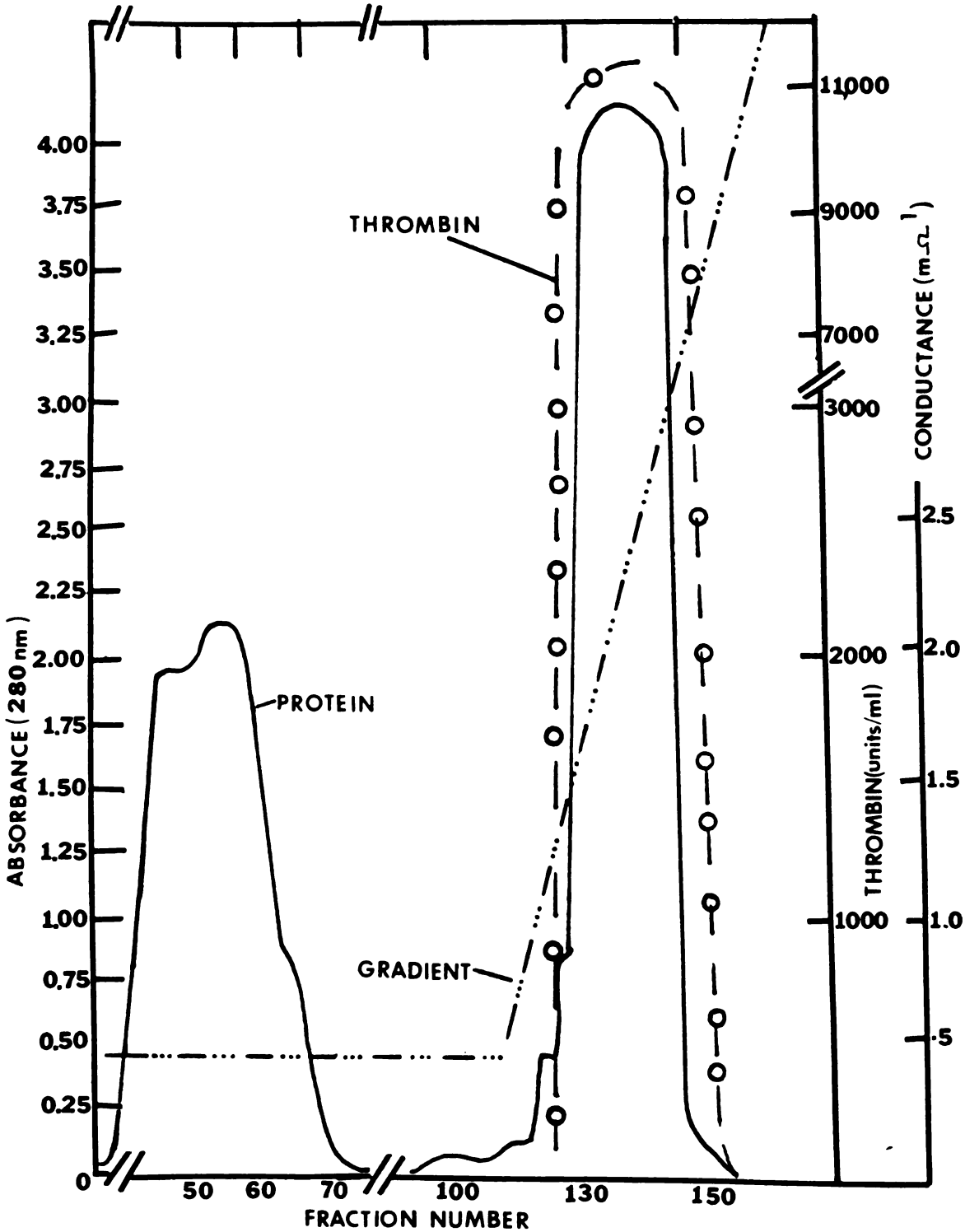

Figure 15. Thrombin chromatographed on Bio-Rex 70

on individual fractions. Specific activity of the peak fractions was calculated to be 3,000 to 4,000 units of activity/mg of protein. The thrombin tubes were dialyzed against VBIS, pH 7.4, and stored at -80C.

Clotting Studies

With the availability of purified coagulants and procoagulants, in vitro testing was begun. An attempt was made to elucidate a coagulant mechanism explaining the hemostatic effect of Factor IX concentrates.

As described in materials and methods, the Factor VIII inhibitor assay is based on a modified APTT.

The first clotting studies were done with Factors X and II (Figure 16). When Factor X, single chain or double chain species, was combined with highly purified Factor II, significant clotting activity was generated. This activity was referred to as Factor VIII inhibitor bypass activity (FEIBA). This activity was the same whether Factor X was purified from a commercial concentrate or purified from cryo-precipitate poor plasma as previously described. Optimal conditions were established by maintaining Factor X at a constant level and decreasing the Factor II activity (Figure 17), or maintaining Factor II as a constant and decreasing Factor X activity (Figure 18). It appeared that maximum generation of FEIBA required approximately a 300%

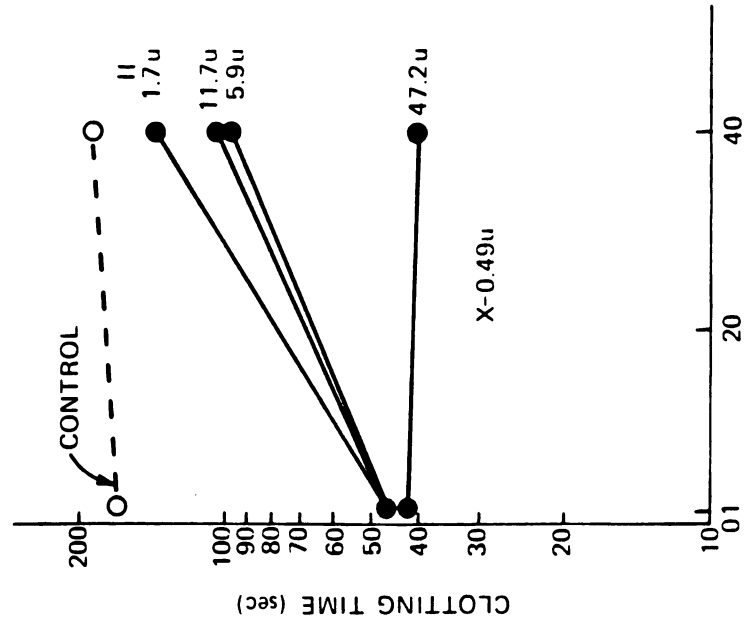

Figure 17. Calculating optimum amounts of prothrombin necessary for FEIBA generation

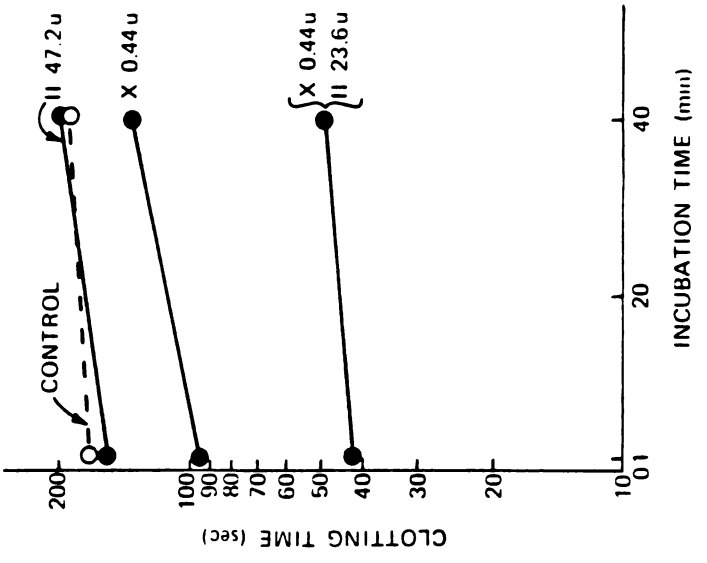

Figure 16. Generation of FEIBA with Factor X + II

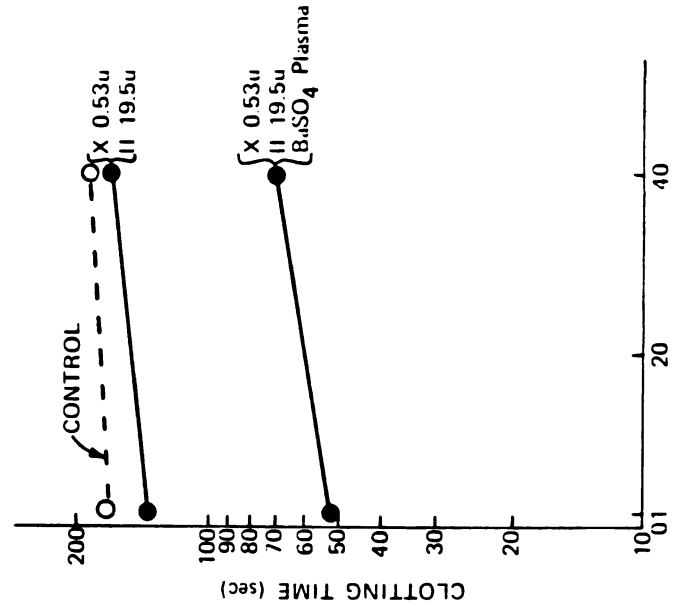

Figure 19. FEIBA assay with depleted Factor V substrate

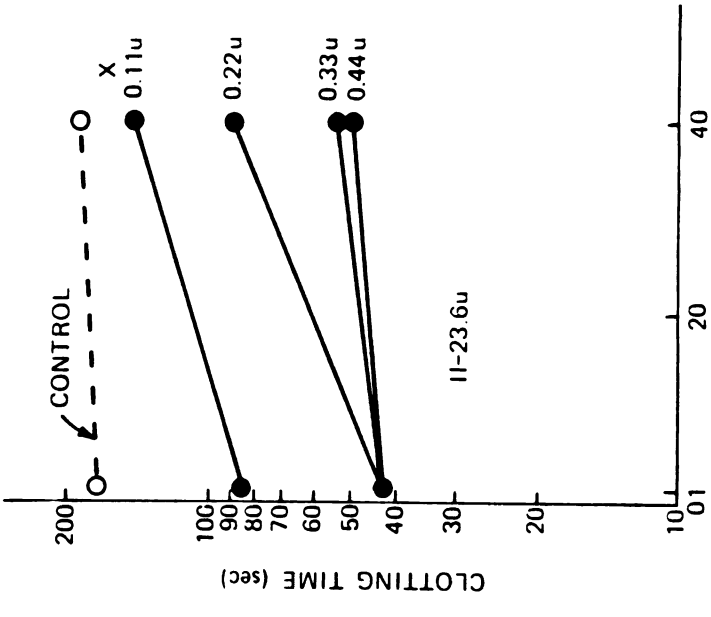

Figure 18. Calculating optimum amounts of Factor X necessary for FEIBA generation

increase in Factor X activity and 100% increase in Factor II activity compared over the levels of these activities in the original Factor VIII inhibitor substrate plasma.

FEIBA was shown to be Factor V dependent by modifying the inhibitor assay in which the inhibitor substrate was depleted of Factor V activity. The inhibitor plasma was incubated at 37C for 24 hours to deplete it of Factor V activity. The depleted substrate (Figure 19) was employed with the addition of Factors X and II, no correction of the abnormal PTT was demonstrated. The addition of $BaSO_4$ treated plasma to the reaction mixture as a source of Factor V completely restored FEIBA coagulant activity.

The question first addressed was whether trace quantities of activated clotting factors, such as Factors Xa or IIa, could be causing the clotting activity. Prothrombin was passed through a small sulphopropyl sephadex column to remove all traces of IIa. Factor X combined with treated Factor II exhibited impaired FEIBA generation when compared to Factor X combined with untreated Factor II (Figure 20). Presumably, the impaired generation was a result of the removal of traces of thrombin. A more direct approach to this problem was achieved by studies using highly purified Factor Xa and IIa. Factor Xa alone or in combination with Factor II failed to generate FEIBA (Figure 21). However, Factor X in combination with IIa (0.05 u/ml) evidenced

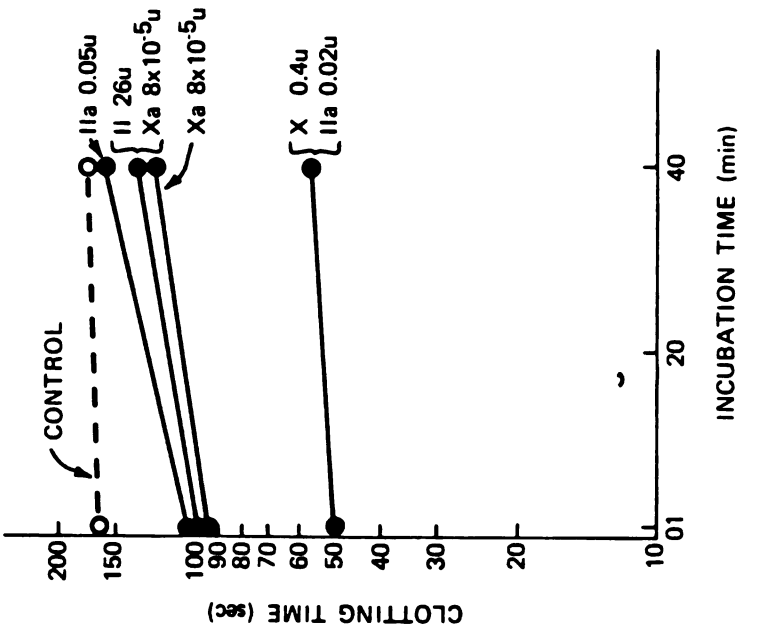

Figure 21. Significant FEIBA generation with X + IIa

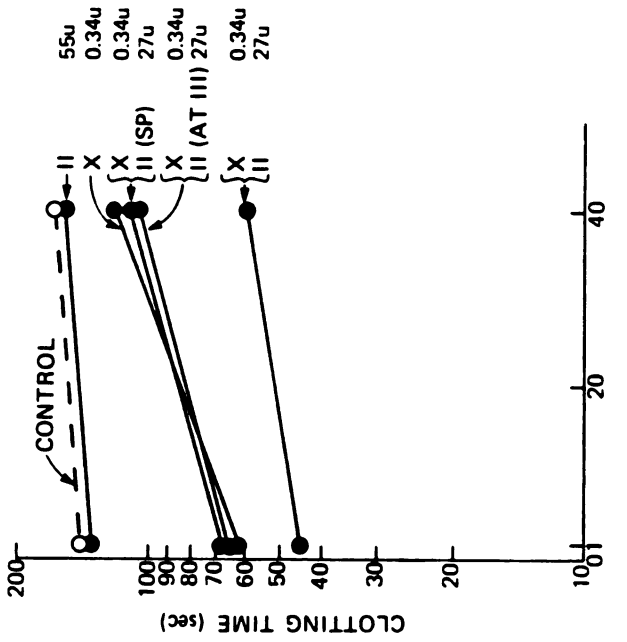

Figure 20. Impaired FEIBA generation

significant FEIBA generation. Thrombin alone was completely inactive.

Factor X, whether prepared by Vician and Tishkoff's method or by Kosow's method, corrected the inhibitor plasma with the addition of trace amounts of thrombin.

A study was undertaken to establish whether the same bypass mechanism would apply to plasma congenitally deficient in a clotting factor. Plasma from patients deficient in Factors VIII, IX, or X were used; the remaining reactants were the same (ACTIN, 0.02M $CaCl_2$). The only addition was $BaSO_4$-treated plasma, as a source of Factor V, to the Factor X deficient assay. A preliminary FEIBA assay was done using Factor X (purified on BDA and then HT, and also Factor X purified on Heparin Agarose, then HT) and IIa. These clotting times pertain only to results after a 2-minute pre-incubation. The results are shown in Table 3 and demonstrate that the by-pass mechanism involves a pathway which enters the coagulation cascade (Figure 1) after Factor VIII. The results are inconclusive with regard to Factor X deficient plasma since reactants contain Factor X.

TABLE 3: The interaction of Factors X and IIa in different congenitally deficient plasma in the FEIBA Assay.

A. Reference assay employing Factor VIII Inhibitor plasma:

	1 minute clot time (sec)	40 minute clot time (sec)
VBIS	125	140
X (.4u*)	77	81
IIa (0.0025 u*)	118	120
X + IIa	35	40

B. Congenitally Deficient Plasma: 1 minute FEIBA

	Factor VIII deficient plasma Clot time (sec)	Factor IX deficient plasma Clot time (sec)	Factor X deficient plasma Clot time (sec)
X**	50	44	53
IIa	77	94	90
X + IIa	47	35	55
VBIS	102	91	95
X***	64	73	52
IIa	87	120	90
X + IIa	61	70	40
Normal Plasma 1110	52	75	41

*Units represent clotting activity in the assay system.
**X purified on BDA, followed by HT column.
***X purified on Heparin Agarose followed by HT column.

Another question addressed was whether X + IIa combined evidence de novo FEIBA activity or whether FEIBA is generated by a time-dependent reaction between Factor X and

IIa. Factor X + IIa were incubated with and without the inhibitor substrate for various time intervals. The substrate employed was the inhibitor plasma from hemophiliac patients. The assays and results are in Table 4 and clearly demonstrate that the generation of FEIBA is not time dependent.

Table 4A: Preincubation of X + IIa with the inhibitor substrate for various time intervals, and proceeding with a standard "1 minute" FEIBA assay.

Incubation Time (min)	Clotting Time (sec)
1	39
3	40
5	50
10	50
15	51
20	60
40	--

Table 4B: Preincubation of X + IIa without the inhibitor substrate for various time intervals, and proceeding with a standard "1 minute" FEIBA assay.

Incubation Times (min)	Clotting Time (sec)
1	39
3	42
5	42
10	62
15	55
20	62
40	62

In a continuation of the time study X + IIa were pre-incubated without the inhibitor substrate for various times, and a "40-minute" FEIBA was done. The results confirm the fact that the X - IIa interaction is not time dependent.

TABLE 5: Preincubation of X + IIa followed by a "40-minute" FEIBA.

Incubation times of X + IIa (min)	40-minute FEIBA clot time (sec)
2	60
40	60
60	61
90	61
120	76

The involvement of Factor IX was examined by utilizing purified Factor IX in the FEIBA assay in place of Factor X. Factor IX was donated by Dr. S. K. Chung, Chapel Hill, North Carolina, and contained 50/ 1 of Factor IX in 0.1M TRIS-HCl/0.15M NaCl and 0.02% sodium azide, 0.1mM Benzamidine - HCl (pH 7.0). Factor IX was dialyzed against VBIS, pH 6.0. FEIBA assays were performed. The clotting times are recorded in Table 6 and indicate that Factor IX is not responsible, in this assay system, for the generation of FEIBA activity.

Table 6: The effect of Factor IX in the FEIBA assay.

	"1 minute" FEIBA	"40 minute" FEIBA
VBIS	145s	150s
X	78s	97s
IIa	90s	111s
X + IIa	56s	66s
IX	103s	107s
IX + IIa	104s	120s

Another in vitro clotting study was done to investigate whether or not human serum, a source of Factors VII, IX, X, XI, and XII, has any combined role in generation of FEIBA. Results of studies employing serum are shown in Table 7.

Table 7: The effects of human serum in generation of FEIBA*.

	"1 minute" FEIBA	"40 minute" FEIBA
VBIS	130s	160s
X	83s	122s
IIa	126s	152s
X + IIa	40s	80s
X + IIa + 0.50 ml 1:10 human serum	40s	80s

*The standard FEIBA assay was used employing Factor VIII inhibitor plasma as substrate.

MOLECULAR STUDIES

As evidenced from the above *in vitro* clotting studies, X + IIa generated FEIBA, but apparently not in a time dependent reaction (see Table 3,4 or 5). The question arises whether X and IIa form a molecular complex with unique coagulant properties *or* Fctor X species had molecularly changed by the action of IIa. The first molecular study was designed so that a X - IIa complex would be readily detected by its exclusion properties from a Sephadex 100 column. The "altered Factor X" possibilities were experimentally examined by testing Factor X with immobilized IIa so that the IIa species would be reserved as reactant after its interaction with Factor X.

In the first experiment a G-100 column, K15/30 was packed and equilibrated with VBIS, pH 7.4. Purified Factor X (Vician-Tishkoff's method) and thrombin (Bio Rex preparation) were incubated for 40 minutes at 37C in a ratio of 1300:1. The mixture was then placed on a G-100 column, and fractions collected at 2.2 ml/tube. Clotting assays for Factors X and IIa were done, as well as 40 minute FEIBA times on the protein fractions eluted from the column. Tube number 19, which was the peak protein tube as well as the peak tube for Factor X activity (4.6 u/ml) exhibited the shortest FEIBA time of 88 seconds after a 40 minute incubation. The void volume for the column was calculated to be 15.2 ml.

Follow-up FEIBA clotting studies were performed to compare the Factor X eluted from G-100 (tube #19) with Factor X not preincubated with IIa or applied to the G-100 column. The results are recorded in Table 8.

Table 8: FEIBA results on X + IIa fractionated off Sephadex G-100.

	"1 minute" FEIBA	"40 minute" FEIBA
VBIS	136s	135s
X DE52 (starting material for G-100 experiment)	130s	150s
IIa Bio Rex	137s	138s
X DE52 + IIa BIO REX	70s	90s
X G-100 #19	63s	87s
X G-100 + IIa BIO REX	40s	55s

SDS gel studies utilizing the treated Factor X plus IIa failed to reveal any Factor X intermediate.

Molecular studies continued with reacting Factor X with immobilized thrombin. The Factor X was obtained from peak 2 BDA and further purified on DE52. Thrombin Sepharose was prepared as previously described. The following reaction mixtures were placed in plastic tubes in an automatic shaking water bath for 40 minutes at 37C:

Reaction A:

0.19 ml X + 0.1 ml IIa-Sepharose + 0.71 ml VBIS (0.4 units)

Reaction B:

0.19 ml X + 0.5 ml IIa-Sepharose + 0.31 ml VBIS (0.4 units)

After the 40 minute incubation, the reaction mixture was separated from the resin using a small disposable column. The supernatant was collected in plastic tubes and assayed as shown below.

	FACTOR X ASSAY	Xa ASSAY	IIa ASSAY
Reaction A	3.2 u/ml	0.01 u/ml	no clot formation at 10'
Reaction B	3.2 u/ml	0.01 u/ml	no clot formation at 12'

FEIBA assays on the supernatants were performed to yield the data listed in Table 9:

Table 9: FEIBA results from the interaction of X with immobilized thrombin.

	1 minute	40 minutes
VBIS	135s	125s
X DE52	49s	95s
X DE52 + IIa	30s	32s
Reaction A 1:1	40s	40s
Reaction B 1:1	54s	97s

From the estimation of IIa attached to the Sepharose (181 units of activity/ml of slurry), Reaction A had approximately 18 units of IIa and Reaction B had approximately 90 units of IIa. This is assuming that all the IIa attached remained active.

SDS gels were run on the two reaction supernatants, and on the Factor X and IIa used in the experiment. The apparent molecular weights for the various bands were calculated and are listed in Table 10.

Table 10: SDS gel results on the interaction of X with immobilized thrombin.

	Reduced	Non Reduced
X^2DE52	85,000 daltons 54,000 20,000	73,000 daltons
IIa	28,000 daltons	32,000 daltons
Reaction A supernatant	73,000 daltons 53,000 19,000	73,000 daltons
Reaction B	80,000 daltons	76,000 daltons
Supernatant	53,000 daltons 20,000	

Attempts to separate out the FEIBA components continued with the preparation of an AT-III Sepharose column. Human AT-III was attached to cyanogen bromide activated Sepharose 4B. Preliminary studies included a reaction with AT-III

Sepharose (2.5 ml of slurry) plus 170 units of IIa. The mixture was incubated at 37C for 30 minutes with constant agitation. The supernatant was then forced through a quick-separation column and IIa assays were performed. Particles appeared at 14 minutes/3 seconds, strands at 15 minutes/45 seconds. The AT-III Sepharose was therefore capable of inhibiting IIa clotting activity.

Purified Factor X and IIa were then incubated for 1 minute at 37C in a ratio of 4:2 respectively. Half of the protein mixture (0.5 ml) was placed on the AT-III Sepharose column, and 0.5 ml was run through a control column of activated Sepharose plus ethanolamine. One minute and 40 minute FEIBAs were done with the following results:

Table 11: FEIBA results of X + IIa incubated with AT-III Sepharose.

	1 minute	40 minutes
Control column supernatant	102s	132s
AT-III Sepharose supernatant	105s	127s
X + IIa before AT-III Sepharose	35s	66s

Reduction and Alkylation of Factor X

Efforts to characterize the mechanism of the inhibitor bypass activity led to an experiment to determine whether the clot promoting activity of FEIBA could be attributed to the double or single chain of the Factor X molecule. The

Factor X was reduced and alkylated as previously described and FEIBA assays were performed. Sodium dodecyl sulfate gels were run to verify proper reduction. The FEIBA clot times are found in Table 12.

Table 12: FEIBA results of reduced and alkylated Factor X.

	1 minute	40 minutes
X (0.4 u)	125s	120s
IIa Bio-Rex (0.0163 u)	142s	162s
X + IIa	62s	70s
X (reduced, alkylated, dialyzed overnight BBS pH 6.0)	142s	139s
X (reduced, alkylated) + IIa	127s	162s

SDS Gel Electrophoresis

Numerous SDS gels were run in an attempt to identify an intermediate which could be responsible for the clot promoting activity in the Factor IX concentrates.

Factor X (peak 2 BDA) further purified on DE52, was incubated with IIa from a BIO-REX column for various times at 37C. Reduced and non-reduced gels were run. Photographs in Figure 22 do show evidence of a Factor X intermediate formed with a X + IIa interaction on two separate occasions.

Attempts to further identify the intermediate or to make certain it was not Factor Xa were unsuccessful due to the presence of Factor IIa in the reaction mixture.

Figure 22. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of Factor X intermediate

In the next experiment, after preincubation of Factors X + IIa, the IIa was neutralized with AT-III. The reaction mixture was then run on SDS gels, the intermediate band cut out, and a FEIBA assay performed. For this experiment, Factor X was purified from Kosow's method, and IIa from a BIO REX column. Preliminary work involved running SDS TRIS gels in order to study the formation of the ATIII-IIa complex (Rosenberg (119)). The lower gel consisted of 7.5% acrylamide. The incubating solutions and reaction mixtures were made according to Rosenberg (119). Approximately 0.8 ml IIa (0.131 mg) and 0.8 ml ATIII (0.128 mg) were combined and incubated at various times, after which 12 ml of the reaction mixture was taken out and applied to the gel. The amount of protein on the gel was calculated to be 5.33g ATIII and 5.43g IIa on each gel. After a 60 second and 2 minute incubation, the bands seen were Complex I (97,000 daltons), Complex II (85,000), ATIII (66,000), and IIa (very light band 32,000). After the 10 minute incubation of IIa + ATIII, the IIa had disappeared from the gel and only the complexes and ATIII bands remained. Therefore, it was possible to run the gels without IIa interference.

Subsequently Factor X and IIa were preincubated for 20 minutes. Then 0.8 ml of the coagulant protein mixture was incubated with AT-III for 20 minutes and SDS gels were prepared. Results of the gels are listed in Table 13.

Table 13: Apparent molecular weights of bands appearing on SDS gels.

		Molecular weights (daltons)
1)	X + IIa Non-reduced	67,000 55,000 34,000 19,000 (contaminant)
2)	X + IIa ATIII	87,000 68,000 56,000 33,200 19,000 (contaminant)
3)	X + IIa ATIII Reduced	95,000 84,000 62,000 49,800 31,500 19,000 (contaminant) 13,500
4)	X Non-reduced	69,000 19,800 (contaminant)
5)	X Reduced	49,000 19,500 (contaminant) 13,400
6)	IIa Non-reduced	38,000
7)	IIa Reduced	33,000
8)	ATIII Non-reduced	58,000
9)	ATIII Reduced	65,000

Chromogenic Substrate Assays

After many *in vitro* studies involving gels, column chromatography and clotting studies, chromogenic peptide assays were undertaken to investigate the kinetics of the coagulant activity. This assay system, in which all components were highly purified, was used in an attempt to elucidate the FEIBA mechanism as well as identify whether FEIBA has amidolytic properties.

S2238, a sensitive and soluble synthetic peptide, was utilized. Numerous chromogenic assays were performed according to the described reaction (Materials and Methods, page 26) or with slight modifications. The source for Factor V was varied and included purified Factor V (SIGMA) or BaSo4 treated plasma.

The addition of trace amounts of thrombin to Factor X, in the assay system, did not consistently accelerate the kinetics of the reaction.

Additional Experiments on Factor X

Ouchterlony Diffusion

The double immunodiffusion technique was employed to test the reactivity of Factor X purified from cryo-precipitate poor plasma with antisera manufactured in rabbits. The precipitate line formed was similar whether the Factor X was purfied from Vician-Tishkoff's or Kosow's method.

One preparation of Factor X was unusual in that it did not exhibit FEIBA activity. However, this particular Factor X was run on the Ouchterlony plates and exhibited the same precipitate arcs against anti-X as the active Factor X preparations.

Isoelectric Focusing

The electrophoretic technique of isoelectric focusing was utilized in an attempt to demonstrate the difference between the two Factor X preparations. Both preparations exhibited an isoelectric point of 4.5.

DISCUSSION

Human Factor X was purified 2000 fold by Vician-Tishkoff's (120) method and approximately 5000 fold by Kosow's (121) method. Both purification schemes yielded a Factor X which was free of other vitamin K dependent clotting factors and relatively homogeneous on polyacrylamide gels. The final products showed 30-50 units of specific activity/mg of protein (Vician-Tishkoff's method) and approximately 100 units of activity/mg of protein (Kosow's method). The molecular weight of human Factor X was consistent with data reported in the literature (122, 123, 124).

Successful activation of purified prothrombin to thrombin correlated well with that reported by Lanchantin, et al (125), and yielded a highly purified thrombin with a specific activity of 3,000 to 4,000 units/mg of protein.

Utilizing the purified procoagulants in the FEIBA assay, it was concluded that the bypassing activity was due to an interaction between Factor X with trace amounts of thrombin. Factor V, calcium, and lipid were essential for

this reaction to take place. Elsinger (126) also concluded that Factor V was essential for the bypassing activity. Both Factor X preparations were equally effective in combining with thrombin to significantly reduce the NAPTT of the inhibitor plasma.

In vitro testing was utilized to examine the interaction of activated clotting factors. The data presented suggests that Factor Xa, by itself or in combination with prothrombin, was not responsible for the clotting mechanism. The data, however, does show a "'Xa-like' activity". This is in contrast to an early report by Kurcyznski (127), stating that Xa could be directly responsible for the bypassing activity. DeWitt and Feinstein (128) also reported that the hemostatic effect was most probably due to activated clotting factors. The clotting assay used in this study however, demonstrated the ability of the bypassing activity to remain after a 40 minute incubation. This is sufficient time for protease inhibitors (Alpha-2-macroglobulin, alpha-1-antitrypsin, AT-III) in the substrate plasma to neutralize Xa. In support of our data, Elsinger (129, 130) presented evidence to exclude Xa as the single factor necessary for FEIBA generation. He treated Xa and Fraction FEIBA (Fraction R) with insolubilized soybean trypsin inhibitor. After 60 minutes, the FEIB-Activity of Xa disappeared while Fraction FEIBA still showed a definitely shortened APTT.

Thrombin was shown to interact with Factor X to produce an impressive reduction in the NAPTT of the inhibitor plasma in this investigation. Thrombin alone was completely ineffective. Elsinger (131, 132) also excluded the involvement of thrombin as the sole factor responsible for FEIBA generation because: (1) the thrombin content of the Prothrombin Complex preparation is too low to shorten the PTT of the inhibitor plasma; and (2) in a controlled experiment, FEIB-Activity of thrombin disappeared completely when treated with insolubilized SBTI, as compared to the FEIB-Activity of treated Fraction R. The activated factor activity of the prothrombin complex prepared in this study was also very low.

The fact that the presumed action of the bypass activity requires or includes more than one factor is also supported by Elsinger (133) and Tishkoff (134). According to Elsinger, gel filtration studies show the molecular species of the coagulant activity to be larger than Factors II, IX and X. He states it is probably greater than Factor IX, but less than 160,000. Tishkoff, in similar gel filtration experiments, located a coagulant that exhibited Factor VIII-like activity in a standard one-stage Factor VIII assay that also had an apparently larger molecular weight.

The effects of purified Factor X and Factor X + IIa to "correct" a plasma congenitally deficient in VIII, IX or X

was seen in data presented in Table 3. It is interesting to note that Factor X alone was quite effective in decreasing the clotting time in comparison to that seen with the buffer alone. Although the addition of IIa to the reaction mixture reduces the time slightly more, it seems that thrombin in these studies may be merely an accelerator of the reaction and not a primary component of the bypass mechanism.

From results of studies of the involvement of the inhibitor substrate in the FEIBA assay, the conclusion is drawn that the substrate is not contributing directly to the active principle of the mechanism. Data are presented in Table 4A which show that incubation with the inhibitor substrate will produce slightly faster clotting times than preincubation without. We assume these subtle differences are merely due to the factors contributed by the substrate to the clotting mechanism: Factor V, fibrinogen, and prothrombin. These will certainly enhance the formation of a clot, but are not the active FEIBA components. These results clearly demonstrate that the X - IIa interaction is not time dependent.

We do not suspect a direct involvement of Factor IX. Contamination with Factor IX would not be significant enough to activate the bypass mechanism (see Table 6).

It can be summarized from the data in Tables 3 through 7 that:

1) The possible contribution of contaminated Factors IX or VIII to the unknown mechanism can be eliminated.
2) The possibility of the inhibitor substrate as contributing the key components (other than V, fibrinogen, and prothrombin) to the mechanism can be eliminated.
3) Purified Factor X shows equally good correction with a plasma congenitally deficient in Factors VIII, IX or X.
4) There is a question as to whether thrombin in the congenitally deficient plasma assay system is indeed involved as the major active principle with Factor X, or is merely an accelerator of the reaction. However, data in Table 6 does support the theory of the X + IIa interaction to produce the most dramatic clotting results.
5) The X - IIa interaction for the generation of FEIBA is not a time dependent reaction.

Assuming the activity of FEIBA is attributable to a coagulant complex formed by the interaction of Factor X and thrombin, molecular studies were designed to isolate the complex by separating out the active complex by column chromatography or by reacting Factor X with immobilized thrombin. Thus, gel filtration would ultimately produce a "pure" active component. Technical problems were

encountered in which FEIBA could not be recovered from the sepharose material (due to dilution effect of the column) or in which thrombin leached off the sepharose and interfered in the FEIBA assay. Thus, these effects were responsible for inconclusive evidence in these sets of experiments.

The theory that the double chain of Factor X contributes to the generation of the bypass activity was supported by the loss of clot promoting activity when the Factor X was reduced, alkylated and then utilized in the FEIBA assay.

Further attempts at characterizing the FEIBA mechanism led to a series of SDS gel experiments. If the molecular model responsible for the bypass mechanism were not a binary complex but rather involved a molecular change such that Factor X would be split by thrombin, it was hoped to show evidence of this new species on SDS gels. Indeed, an intermediate band was seen on several gels but could not be exclusively identified as a X intermediate (X or X). The possibility remained that it might be Factor Xa. The interference of thrombin in the gel system was successfully eliminated by electrophoresing an AT-III-thrombin complex. However, results did not firmly indicate a new molecular species.

Although purified <u>in vitro</u> clotting systems revealed a coagulant complex formed by the interaction of Factors X, V and thrombin to be responsible for the bypass mechanism, it

was necessary to pursue a biochemical approach. Relating FEIBA to kinetic parameters involved utilizing purified procoagulant factors in a synthetic chromogenic substrate assay to evaluate the clot promoting activity.

Elsinger (135) briefly compared the amidolytic activities of FEIBA IMMUNO with those of thrombin and Factor Xa. His conclusion was that neither Factor Xa nor thrombin could be solely responsible for FEIB-Activity. The reaction employed in this study, however, was more involved and was aimed at proving that Factor X and IIa, in a purified system, were capable of the generation of prothrombinkinase, independent of the intrinsic or extrinsic cycle. Evidence of increased kinetics with the addition of trace amounts of thrombin to the chromogenic assay system was sought. However, data collected was not sufficiently reproducible to substantiate the data collected from the in vitro and clotting assays.

Attempts at characterizing the mechanism of Factor IX concentrates involved examination with plasma factors only. Evaluation of the enhancement of Factor IX concentrates with platelets, as reported by Vermylen (136), was not investigated in this study.

CONCLUSION

A modified activated partial thromboplastin time was devised to assay for Factor VIII inhibitor by-passing activity (FEIBA) of Factor IX Concentrates. The activity was based on the correction of the abnormal activated partial thromboplastin time of Factor VIII Inhibitor plasma after a one minute and forty minute incubation time. The procoagulant and coagulant proteins employed in the bypass assay were relatively homogeneous and were obtained from the prothrombin complex by biochemical fractionation. FEIBA, in our *in vitro* clotting system was found to be dependent on the interaction of Factors X, IIa and V. It was not established whether these proteins are reacting as a binary complex or if thrombin is merely an accelerator of the reaction.

While not enabling us to characterize an exact mechanism, utilizing the chromogenic substrate assay

provided a technique for analysis of the kinetics of the reaction.

In attempting to characterize the mechanism of Factor IX concentrates, testing was developed in purified systems. Although purification was presumed to create optimal conditions, DeWitt and Feinstein (137) reported to the contrary. They reported that the more the concentrates were "cleaned up" the less effective and less potent they became.

Current state of knowledge does not allow designation of the exact mechanism of the by-pass activity. Although data shows strong evidence for the direct involvement of Factors X and IIa, further investigation into the biochemical mechanism is necessary.

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