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APPARENT PROTHROMBINASE ACTIVITY OF HUMAN FACTOR X: A MECHANISM FOR THE FACTOR VIII INHIBITOR BYPASSING ACTIVITY OF PROTHROMBIN COMPLEX CONCENTRATES

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APPARENT PROTHROMBINASE ACTIVITY OF HUMAN FACTOR X: A MECHANISM FOR THE FACTOR VIII INHIBITOR BYPASSING ACTIVITY OF PROTHROMBIN COMPLEX CONCENTRATES

By

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This dissertation is dedicated
to my wife Suzanne and to my children.
They endured my anxieties and frustrations
and have continued to give me
their love and support.

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CHARACTERIZATION OF THE FACTOR VIII ANTIBODY

Introduction

Antibodies to factor VIII have been demonstrated in two distinctly different patient populations: Inhibitors that develop in patients with inherited severe hemophilia A (factor VIII defeicency) and inhibitors that arise spontaneously in non-hemophilic patients such as, postpartum complications, certain immunologic disorders and in older individuals as a spontaneous complication. These clinical subgroups must be kept in mind when the antibody response to factor VIII is being evaluated. Although the antibody produced in both cases is usually of the immunoglobulin G class, there appears to be some difference in subclass type, light chain specificty and in the reaction kinetics of the antibodies produced. In hemophilia A second order inactivation kinetics are more common while more complex kinetics with incomplete inactivation is characteristic of antibodies developing in non-hemophilic patients.
Both of these characteristics will be discussed in more detail.

Three important considerations regarding antibodies to

factor VIII include the incidence, properties and etiology of the factor VIII inhibitor. Because of the size and complexity of the factor VIII molecule there are a great number of potential antigenic determinants. For this reason the characteristics of the factor VIII inhibitors, although not unique, are extremely complex. As of yet, no definitive answer has been found that thoroughly explains the etiology and characteristics of the immune response to factor VIII.

Incidence

It is important, when discussing the incidence of factor VIII inhibitors in the hemophilic population, to remember that development of an antibody is the result of multiple immunizations with an isologous plasma protein component in response to the need for replacement therapy. Therefore, the incidence of antibody development is correlated with the total number of exposure days to factor VIII prior to its appearance. In light of this, various statistics on the incidence of factor VIII inhibitors in different age groups and between the "at risk" population of severe hemophiliacs (< 1% factor VIII) and those with a mild deficiency (2% to 30% factor VIII) will be discussed.

In 1975 Ruggeri reported an inhibitor incidence of 13% in the "at risk" population of hemophiliacs (1). However, when broken down into the age groups 0-9, 10-24, and over 25 years of age the percentages for the same "at risk" population were 22%, 12% and 6% respectively. This

suggested an increased risk at a younger age. If,however, these results were evaluated strictly on the basis of the number of exposure days to factor VIII prior to the development of an inhibitor "predisposed" individuals would be selected out at a younger age and the value of 6% in the 25 and over age group would reflect a statistically biased figure. In a report on the incidence of inhibitors among hemophiliacs a value of 14.6% was given for the incidence of inhibitors in patients under 10 years of age as opposed to an overall value of 7.8% for the total group studied (2). However, in a large national study Biggs reported an incidence of only 4.1% for the same age group (3). Again, it would be valuable to relate total number of exposure days prior to development of an inhibitor in both of these reports. Likewise, the incidence that has been reported based on the difference between a severe and mild hemophiliac would be more meaningful if evaluated in terms of the total number of exposure days. Despite the variability in the reported incidences of inhibitors to factor VIII the majority of these inhibitors occur in severe hemophiliacs with a frequency of 5 to 18%.

Antibody Characteristics

Based on the molecular sieving characteristics on Sephadex gel, the fractionation pattern on DEAE cellulose, the ability to be precipitated the protein with high salt concentrations, the electrophoretic pattern and the ability to neutralize the inhibitor activity with antisera specfic

for IgG, inhibitors to factor VIII have the same immunochemical properties as other immunoglobulins of the IgG class (4). Initial work on the antibody structure of inhibitors to factor VIII suggested that the apparent homologous antibodies were of monoclonal origin (5,6). They were identified as having a single kappa light chain specificity and a similiar homogeneity of the heavy chain, specfically IgG_4. This heavy chain subclass is consistant with observation that the factor VIII antibodies do not fix complement. More recently a limited number of patients demonstrating a combined light chain population of both kappa and lambda and a mixture of predominately IgG_3 and IgG_4 heavy chain subclasses have been reported (7,8). This heterogeneous population of IgG antibodies appears to be more characteristic of cases of inhibitor development in non-hemophilia A patients although a broad generalization should be avoided. Table 1 is a summary of data compiled from several different studies on the structure of the factor VIII antibodies. Although the majority of the inhibitors are IgG in nature and exhibit restricted heterogeneity in heavy chain subclass and light chain type, IgM antibodies have been reported particularly in the non-hemophilic population (9).

The majority of the antibodies produced in response to factor VIII are specific for the procoagulant portion of the factor VIII molecule and have no effect on other clotting factors (10). The inhibitors do not interfere with the

other functional activities of the factor VIII complex. Factor VIII related antigen and bleeding times are equivalent to the values found in hemophiliacs without an inhibitor. There is normal platelet retention on glass beads and normal ristocetin aggregation and ristocetin cofactor activity. In addition, factor VIII inhibitors show a great deal of species specificity. For example, bovine and procine factor VIII are only partially inhibited by antibody to human factor VIII and as a result hemophiliacs with an inhibitor can be treated effectively with factor VIII from these animals (11). As would be expected, however, antibodies to the animal protein will develop and, as a result, antibodies with two specificities can then be demonstrated in the human serum.

Table 1. Structural Characteristics of Factor VIII Antibodies.

		Un-	IgG			Light Chains		
Patients	Number	specified	1+4	3+4	4	k*	1*	k+1
Hemophilic	15	7	5	3	-	10	1	4
Nonhemophilic	14	11	2	-	1	6	2	6
Hemophilic or nonhemophilic	10	10	-	-	-	10	-	-

*k = kappa, 1 = lambda
From: Mariani, Russo and Mandelli (12)

Antibodies formed in response to factor VIII are non-precipitating. This may be due to the fact that the factor VIII:C is present in too low a concentration to form a visible precipitate and/or either the antigens are monovalent or the antigenic determinants lie far apart and

are therefore not capable of forming a lattice to permit precipitation. The fact that VIII:C can be measured by a two-site immunoradiometric assay would rule against the possibility of a monovalent antigen. Finally, as is the case in the production of monoclonal antibodies, the more restricted the antibody response the less likely it is to form a precipitate.

Development Of An Inhibitor

A major question that remains unanswered pertains to the mechanism responsible for the development of a factor VIII inhibitor and why only 5 to 18% of the multitransfused hemophiliacs develop an antibody. Meyer proposed two hypotheses that might explain the genesis of factor VIII antibodies (13). The first is based on the genetic control of the general immune response and involves antigen recognition, macrophage processing and antibody production. It is known that hemophilia may occur as a result of either suppression of VIII:C production in which case there is a lack of cross reacting molecules (CRM-), or may result from production of a defective protein (CRM+). It was felt that antibody production would be confined to the CRM- group because they would be most likely to respond to the foreign protein. This explanation did not, however, agree with the observed statistics showing that 80% of hemophiliacs are CRM- and only approximately 10% of these develop an antibody. Alternatively, there may be allotypic forms of the factor VIII molecule. This particular explantion remains speculative because the assay systems are not sensitive

enough, at present, to detect single amino acid variations that could account for the allotypic differences. Finally, Meyer suggested, in a second hypothesis, that all severe hemophiliacs show an immune response to factor VIII in the form of low titer and low affinity IgM antibodies that would not be detected. In most cases the immune reponse would be suppressed but in 5 to 18% the IgM response would switch to IgG synthesis.

In 1977 Frommel and Allain, in order to determine whether a familial predisposition to become immune to factor VIII did or did not exist, presented their accumulated data on a number of family groups genetically disposed to factor VIII deficiency (14). Twenty five sibships were investigated with an overall incidence of factor VIII antibody of 46%. Of these 25 sibships, concordance for responsiveness was found in 19. Only 6 brother pairs demonstrated nonconcordance. This data suggested that genetic factors could be involved in determining immune responsiveness to factor VIII. If linkage between the immune response to factor VIII and the major histocompatibility complex (MHC) could be shown this would establish genetic control of the antibody response to factor VIII. Frommel et al. reported on HLA antigens and factor VIII based on the following assumption for linkage:

> If the ability to develop antibody to factor VIII is dependent on the inheritance of a dominant MHC-linked gene, then it would be expected that hemophilic siblings with antibody to factor VIII will share one or two HLA haplotypes, and that non-concordant sibs (responder - non-responder) will differ in one or both

haplotypes, unless a cross-over had occurred during meiosis (15).

They concluded that although the magnitude of the immune response is under genetic control there was insufficient evidence to support linkage with the MHC and, in addition, that immunity to factor VIII "does not express preferential association with HLA specificities."

Kinetics Of The Inhibitor Response

Two additional observations regarding the characteristics of the factor VIII antibody should be mentioned because of the effect they have on determining treatment and evaluating inhibitor assay results. The first of these bears directly on the antibody titer in response to immunologic challenge during administration of factor VIII concentrates. It has been noted that the population of inhibitor patients can be divided into high and low responder groups with an observed high/low ratio of approximately 4:1 (16). These groups are based on the degree of anamnestic response observed as a result of therapy with factor VIII. In the high responder group antibody is discovered after 2 to approximately 50 cumulative exposure days to factor VIII and antibody titer is greater than 5 Bethesda Units and may, upon challenge, go as high as 1000 to 2000 Bethesda Units. In the low responder group antibody does not usually appear until after 40 to 200 cumulative exposure days and anamnestic response is minimal and the antibody may disappear. It should be

noted when discussing treatment that low responders can be given factor VIII although larger doses are required.

The second characteristic of the factor VIII inhibitor relates to the inactivation kinetics mentioned previously in this section. Not only has a difference in the characteristic response of the factor VIII inhibitor to antigen challenge been noted but, in addition, two inactivation patterns representing two types of antibodies have been distinguished by kinetic analysis (17). Type I antibodies have a linear relationship when the log of residual factor VIII activity is plotted vs. the concentration of the antibody. If the concentration of antibody is sufficient it will completely neutralize the factor VIII:C activity. Type II antibodies, on the other hand, show a nonlinear relationship when plotted under identical conditions and do not completely neutralize the factor VIII:C activity.

Biggs et al. characterized the factor VIII response to both types of antibody (18,19). In the case of Type I reaction they concluded that...

> antibodies which give more or less straight line concentration graphs when drawn on log/linear paper may be regarded for practical purposes as if they underwent an irreversible second-order reaction with factor VIII and that even when antibody is not present in excess the concentration graph can be used to give a relative measure of antibody concentration.

In the case of Type II antibodies, although they were able to develop a mathematical model to fit the more complex kinetics of these antibodies, a specfic explanation for their mode of action was lacking. It was felt that one possible mechanism would involve an antibody factor VIII complex that

retained activity. An additional hypothesis on the action of Type II antibodies was suggested by Gawryl and Hoyer (20). They suggested that the reduced factor VIII:C inhibition could be due to steric interference from the von Willebrand factor portion of the molecule. They found that when factor VIII:C activity was seperated from the von Willebrand factor the inhibition kinetics were no longer complex but resembled those of a Type I reaction.

Characteristically Type I antibodies show a fairly rapid inactivation of the VIII:C activity which reaches a plateau and upon further addition of factor VIII no additional neutralization is observed. However, type II antibodies demonstrate an initial rapid inactivation followed by a slowing that does not plateau. Upon addition of more factor VIII there is continued slow inactivation (18,19).

The Type II reaction is most common in nonhemophiliacs and, according to Allain and Frommel (17), these antibodies have less affinity for factor VIII than those that arise in treated hemophiliacs. The implication in treating Type II individuals lies in the ability to express some factor VIII:C activity even in the presence of excess antibody (21-24). Finally, Croissant et al. demonstrated the presence of two distinctly different antibody populations in patients with autoantibodies to factor VIII indicating at least two different antigenic determinants that are recoginzed in vivo (25).

INHIBITOR ASSAY TECHNIQUES

A number of assay techniques have been devised to quantitate the level of anti-factor VIII:C. These assays range from inhibition of factor VIII:C activity, as in the New Oxford and Bethesda assays, to immuno-radiometric assays (IRMA). Based on the characteristics of the inhibitors to factor VIII one could deduce that the variation in kinetics, which could be a reflection of a decreased affinity for the factor VIII molecule and/or different antibody populations to varying antigenic determinants, could very likely cause difficulty in determining antibody levels. In addition to the inhibitor kinetics, four additional methodological aspects of the inhibitor have been identified by Lechner and must be kept in mind when evaluating inhibitor assays (12). These include: 1) sensitivity; 2) compatibility; 3) reproducibility; and 4) the time course of inactivation. The time course of inactivation refers to the fact that in some patients the inactivation is very rapid, 30 minutes, while in most instances it takes several hours.

The measurement of inhibitors by coagulant activity is presently performed by one of two different assay techniques. In 1975 the National Heart and Lung Institute sponsored a meeting to promote the standardization of the measurement of factor VIII inhibitors (26). The proposed assay (Bethesda assay) uses normal pooled citrated human plasma as a standard source of factor VIII and is most accurate in the range of 25% to 75% inhibition. Higher

inhibitor levels are diluted to fall in this range. However, more sensitive methods must be found to determine inhibitor levels lower than 25% but still of clinical significance (27). In this assay a test sample producing a residual factor VIII activity of 50% is considered to contain one Bethesda Unit (BU) of inhibitor/ml. The units are arbitrary and do not imply any particular level of factor VIII concentrate that, when infused, would neutralize the antibody.

The second method is the New Oxford technique. The New Oxford method differs mainly in the use of factor VIII concentrate as a standardized source of factor VIII, as opposed to normal human plasma. Table 2 outlines both the New Oxford assay and the Bethesda method.

Austen reported on a comparative study of the New Oxford technique vs. the Bethesda method (12). Eleven laboratories from different countries were involved in the study and the following conclusions were drawn:

Table 2. New Oxford and Bethesda Antibody Assays

	New Oxford	Bethesda
Source of Factor VIII	VIII Concentrate	Plasma
Amount of Factor VIII	0.5 to 1.0 U/ml	50% of plasma
Incubation period (hrs)	4	2
Antibody Units	1 unit destroys 0.5 units of VIII	1 unit destroys 50% of factor VIII added

From: Mariani, Russo and Mandelli (12)

1. There is excessive disagreement between laboratories in antibody measurement.
2. This between-laboratories error is the greatest component of the total errors.
3. Generally, laboratories ranked antibody samples in the same order.
4. Considerable improvement (30-40%) would be obtained if a standard antibody were available.
5. In antibody assay, particularly the Bethesda method, a fixed starting level of factor VIII should improve agreement.

It should be kept in mind that these assays do not reflect the complex kinetic reactions seen in most patients with autoantibodies to factor VIII. It has been suggested that in the case where several dilutions yield a level of 50% of the control the least dilution giving a 50% value be used to calculate the results (12).

Most recently, the results of an IRMA have been reported by Peak et al. and Furlong et al. (28,29). The results, although comparable to the Bethesda method yet more sensitive, vary somewhat based on the high titer anti-VIII antibody used as a standard. This is probably due to a slight variation in affinity as the dilution curves are parallel when percent inhibition is plotted versus the log dilution of antibody.

THERAPUTIC IMPLICATIONS

The development of an inhibitor to factor VIII presents obvious therapeutic complications and although there tends to be some agreement on the particular course of treatment, there is still considerable disagreement on the best method of preventing an anamnestic response. The treatment protocols presently being used will be briefly discussed and a major emphasis will be placed on the use of the prothrombin complex concentrates (PCC) because of the central role that PCC's played in initiating this work.

The recommended therapy varies depending on whether the patient is a high or low responder, whether the antibody developed as a result of an inherited deficiency of factor VIII or as an autoantibody, the severity and location of the bleed and the initial inhibitor titer. A number of clincians feel that, in all cases, the preferred method of treatment is factor VIII concentrate unless the antibody titer is sufficiently high to dictate against this. Additional courses of treatment include plasmapheresis to remove the inhibitor, factor VIII replacement with concomitant administration of immunosuppresants designed to minimize the immune response, use of nonhuman (procine or bovine) factor VIII and the use of either "non-activated" PCC's or "activated" PCC's both found to be capable of bypassing the need for factor VIII.

Immunosuppression

Most often immunosuppressive therapy is used in the case of autoimmune inhibitors in non-hemophilic patients (30, 6, 31). Hultin et al. suggested that the major contributing factors to the effectiveness of immunosuppressive therapy are the titer and duration of inhibitor prior to initiation of therapy and the degree of factor VIII exposure between inhibitor appearance and attempted immunosuppression (32). Although there have been reported incidences of prevention or decrease in the anamnestic response when immunosuppressive therapy is used the majority of the cases of hemophilia A have not responded to immunosuppressive therapy (33,34). The use of immunosuppressive therapy must be evaluated carefully because of the long-term toxicities of these drugs (35).

Plasmapheresis

Plasmapheresis appears to be a viable option but only in a limited number of cases and only under certain circumstances. Considerations include: 1) material to be used in the exchange process, 2) the inhibitor titer and 3) the anamnestic response. Plasmapheresis appears most effective when the inhibitor titer is low and appears not to work when there is an anamnestic rise in the antibody titer. In addition complications associated with plasmapheresis have been anaphylactic reaction, headache, fever, vomiting and hepatitis and several cases of sudden death have been

reported (12). In addition to the conventional use of plasmapheresis antibodies have been removed by passing the plasma fraction over a sterilized protein A sepharose column prior to returning the plasma to the patient. This procedure selectively eliminates the IgG fraction and precludes the need to use fresh frozen plasma to maintain adequate hemostasis.

Immunotolerance

In 1981 Brackmann and Egli reported the use of high doses of factor VIII concentrate in conjunction with activated factor IX concentrates to treat hemophiliacs with inhibitors (36). The intent of this procedure is to suppress the inhibitor production by inducing immunotolerance. Their protocol consisted of administration of 75-100 U/kg of factor VIII concentrate and 40-60 U/kg activated PCC's twice daily until the inhibitor level fell below 0.5 BU/ml. At this point, the therapy was altered to a regimen in which the same doses were alternated between twice a day and once a day. When the inhibitor titer fell below 0.2 BU/ml each product was given only once daily until inhibitor levels were undetectable. The use of activated PCC's was then discontinued but factor VIII administration was sustained until the in vivo half-life of factor VIII was normal in the treated patients. Additional work using alternate protocols has been reported (37-39). Recently, White et al. reported the effective use of continuous factor VIII administration, without the use of activated PCC's, in

reducing a high-titer inhibitor (40). Most recently Aznar et al. reported on the continuous use of factor VIII concentrates and fluprednisolone to suppress the immune response. The corticoid was used as an immune suppressent (41).

The main problem with these treatment regimens is the necessity for an extremely large amount of factor VIII. This becomes a drain on limited resources, and is an extremely costly procedure. White et al. (40) reported the use of 5,695,230 U of factor VIII in one patient totaling approximately $626,500 in the 56 month course of treatment and this figure does not reflect the additional cost of the activated PCC's described in the original protocol of Brackmann and Gormsen (42).

Porcine Factor VIII

Because of the minimum degree of cross-reactivity between human and porcine factor VIII and because the porcine product has been highly purified to eliminate adverse reactions the product has been used used to treat high titer factor VIII inhibitor patients (43). Recently, Fatti and Mannucci reported on the use of a polyelectrolyte fractionated procine factor VIII to treat hemophilia A. Although there were problems with thrombocytopenia, resistance and anamnestic rises in antibody titer it was concluded that the use of the porcine product was a rational and effective therapeutic choice when one is faced with antibody titers above 10 U/ml and difficult clinical

situations (44).

PROTHROMBIN COMPLEX CONCENTRATES

In 1975 the results of a survey on the methods of preparation of Factor IX concetrates was reported by the Task Force on the Clinical Use of Factor IX Concentrates (45). Two major types of product were identified: 1) those containing mainly II, VII, IX, and X, and 2) those containing II, IX and X and being essentially free of factor VII. The products were prepared from a variety of starting material including: fraction I supernatant, cryoprecipitate supernatant, fraction III, fraction IV-1 and precipitate P. In addition both citrate and EDTA have been used as the anticoagulant. The three absorbents most often used included DEAE-sephadex, DEAE-cellulose and tricalcium phosphate. The major difference in the absorbents relates to the content of the final product. Products prepared using tricalcium phosphate contained factor VII and those prepared with DEAE-sephadex or cellulose either did or did not depending on the isolation conditions.

Generally, for any given product it was found that the potencies of II, IX and X were approximately equal and for products containing factor VII the ratio of coagulant/procoagulant proteins was usually between 20 and 30 U/ml. Additional information on techniques for the preparation of PCC's is available (46). Recently the use of "activated" products has gained widespread interest and the two products most often used are Immuno FEIBA® (Vienna,

Austria) and Autoplex® (Hyland Laboratories). These particular products contain a greater amount of the activated form of the vitamin K dependent enzymes. Table 3 is a list of both the major protein constituents in the PCC's as well as minor contaminants that have been identified.

Testing Procedures

A number of assays have been devised to quantitate both the in vitro and in vivo potency of the PCC's. Although several of the in vitro assays correlate roughly with what can be expected to occur in vivo and allow the clinician to estimate a range of effective dosages, none of the in vivo assays correlate well with the observed clinical

Table 3. Protein Constituents of Factor IX Concentrates

Major	Minor	Misc.
Factor II	IIa	Antithrombin III
Factor X	Xa	Phospholipid
Factor IX	IXa	
Factor VII	VIIa	
	XII	
	XI	
	VIII	
	V	

improvements seen as a result of the Factor Eigth Inhibitor Bypassing Activity (FEIBA). Table 4 lists a number of the assays that have been used as biological probes to assess FEIBA.

For a comprehensive review of the assays available for

determining bypassing activity the reader is referred to Prouse et al., Prouse and Pepper, and Sar et al. (47-49).

Table 4. Biological Probes for Assessing FEIBA*

NAPTT*	Rabbit Stasis Model**
FEIBA	Rabbit Non-Stasis Model+
TGt_{50}	King Test
Recalcification time	Vermylen Test

*Kingdon et al. (50)
**Wessler et al. (51)
+Prowse and Williams (52)

Adverse Reactions

Two particular issues are of major concern as a result of administration of PCC's to either hemophilia B patients or hemophilia A individuals with an inhibitor. The first of these involves the risks of hepatitis and most recently the accompanying possibility of the transmission of the causitive agent of acquired immune deficiency syndrome (AIDS). The second relates to the observed thrombogenic complications associated with administration of PCC's.

Hepatitis

The incidence of reported cases of hepatitis among users of PCC's is fairly high (53). Aronson reports that....

> while commercial products made in the USA have been most frequently involved in the published reports of hepatitis, it would seem that this is in fact a universal problem.

A survey conducted by Hoofnagle et al. on the incidence of serologic markers indicating prior infection with hepatitis

B in patients being treated with PCC's revealed that the overwhelming majority were positive (54). Although even the most sensitive test cannot always detect 100% of the plasma units positive for hepatitis B antigen it is important that pooled plasma products be thoroughly tested.

Hepatitis testing falls into three catagories that are differentiated based on the sensitivity of the assay. First generation testing includes Ouchterlony agarose double diffusion (AGD) which has a high degree of specificity but a low sensitivity. Second generation tests include counter immunoelectrophoresis (CEP), complement fixation (CF) and inhibition of passive hemagglutination (IPH). These tests are more rapid and 5 to 10 times as sensitive as AGD. It should be stressed that neither AGD, CEP or CF consistently detect HBsAG when present in relative low concentration. Third generation tests include radioimmunoassay (RIA), reversed passive hemagglutination (RPH) and the agglutination flocculation test (AFT). Testing with third generation tests provides approximately 100 to 1000 fold greater sensitivity than AGD.

Acquired Immunodeficiency Syndrome

Besides the obvious risk of hepatitis that is associated with receiving blood components from large batches of pooled plasma, there has been a great deal of concern recently over the possibility of contracting AIDS via the same mechanism. To date, there have been

approximately 60 cases of AIDS reported within the hemophilic population. A recent article by deShazo et al, found that the ratio of helper inducer lymphocytes (OKT4+) to suppressor-cytotoxic T-lymphocytes (OKT8+) was depressed in both hemophilia A and B patients receiving factor VIII concentrates and those receiving factor IX concentrates (55). In addition, it would be interesting to know what percent of the normal population, if any, have an abnormal OKT4+/OKT8+ ratio so that a more clear cut diagnostic implication could be associated with helper-suppressor ratios. Most recently a virus (human T-cell lymphotrophic retrovirus subgroup III), thought to be the causative agent of AIDS, has been isolated and a test has been developed to facilitate the wide spread screening of blood donors (56,57). In a population of Swiss patients studied by Schupbach et al. 100% prevalence of antibodies to HTLV-III was found in individuals with AIDS or pre-AIDS, a prevalence of 25% or greater in the known risk groups and no antibody-positive cases were found in 83 healthy donors (58).

Thrombotic Complications

Thrombotic complications have been reported as a result of administration of factor IX concentrates. These have included thrombophlebitis, localized arterial and venous thrombosis, disseminated intravascular coagulation (DIC) and pulmonary emboli (59-65). In addition, Gruppo et al. and Agrawal et al. have reported the development of fatal myocardial infarction following the administration of

PCC's (66,67). Whether or not the thrombogenic character of these concentrates is in anyway related to FEIBA has not been determined. This particular issue will be dealt with in more detail when the possible mechanisms of the bypass reactions are discussed.

Clinical Efficacy Of Treatment With Prothrombin Complex Concentrates

The majority of the reports on the efficacy of treatment with PCC's have been anecdotal in nature and a good deal have dealt mainly with the treatment of hemarthrosis. In addition variations in lot to lot potency of the complexes and intermanufacturer variations in producing the complex have made clinical assessment more difficult.

Based on manipulations during the preparation of PCC's there are two different products presently available for the treatment of hemophilia B and hemophilia A with a inhibitor to factor VIII. Broadly defined, these products fall into the classification of either a "non-activated" or "activated" PCC. The "non-activated" products are prepared according to the methodology previously cited in this review. Until recently, however, the specific mechanism by which the "activated" products are prepared was not known. Information regarding the specific mechanism by which these products are manufactured, although protected by patent rights, is now available (68,69). Mitru et al. (68) utilize two different methods for producing FEIBA in the PCC's. Method A involves adsorption of Effluent I from a Cohn

fractionation onto DEAE-Sephadex®, subsequent elution of factors II, VII, IX, and X and finally exposure to free Ca^{+2} concentrations in the range of 0.5 to 0.8 mM per liter of eluate. The activation procedure takes place usually for a period of about 24 hours at controlled pH and temperature. Method B is simply a modification of the elution characteristics used to remove the prothrombin complex from the DEAE-Sephadex®. In this case all factor concentrations are kept at >10 U/ml and the eluate is simply maintained at a suitable pH and temperature during which time FEIBA is spontaneously generated. Both these products are reported to be substantially free of IIa (< 1.5 U/ml) and factor Xa with ratios of FEIBA to IIa and Xa of at least 50:1 and 45:1 respectively. In an alternate method FEIBA is generated by first activating citrated plasma with a contact activator, such as kaolin, and then isolating factors II, VII, IX and X by chromatography on a basic ion exchanger such as DEAE (69).

Because the efficacy of PCC's for the treatment of inhibitors has been questioned, double blind studies have been conducted using both "activated" and "non-activated" products. The first of these studies was reported by Lusher et al. in 1980 (70). In this study Konyne and Proplex, both "non-activated" products, were tested for their effectiveness in treating acute hemarthrosis of the elbow, knee or ankle as opposed to an albumin placebo. Results were based on the examination of patients with both objective and

subjective criteria six hours after administration of either the placebo or prothrombin complex concentrate. Subjective data included the degree of joint pain. Objective data was based on joint circumference and the measured degree of mobility. Their findings indicated that the overall perceived effectiveness was 28.6% for the albumin placebo, 48.1% for Konyne and 53.2% for Proplex. There was a significant difference ($P<0.0001$) between the concentrates and the placebo but no statistical difference between the two concentrates.

Subsequently, Sjamsoedin et al. reported on a double-blind clinical trial on the use of FEIBA, an "activated" product, as opposed to Prothromblex, a "non-activated" product (71). The study included mucocutaneous bleeding, joint bleeding and muscle bleeding and, the subjective and objective criteria used for assessment were similar to those used by Lusher et al. In this case, the Prothromblex was effective in 52% of the cases evaluated and the FEIBA product was found to be 64% effective. The value of 52% for the "non-activated" product agrees well with the work of Lusher et al. and although the "activated" product showed an additional 12% improvement, without more extensive comparisons it is difficult to say with certainty that there is a statistically significant difference between the "activated" and "non-activated" product.

Most recently Lusher reported on the trial results of Autoplex ("activated") and Proplex ("non-activated") at the IXth International Congress on Thrombosis and Haemostasis

(72). In this particular trial no significant difference was found between the use of the "activated" vs. "non-activated" prothrombin complex concentrate.

The fact that the prothrombin complex concentrates are effective has, therefore, been established. This refutes the observations that the products were losing their effectiveness (73-77). Whether or not the "activated" product is equal to or better than the "non-activated" product is difficult to ascertain with certainty. The major problem lies in the large variation between the clotting factor concentrations in each product. Table 5 shows the results of the factor assays on two lots each of Autoplex® and Immuno FEIBA® obtained by Abildgarrd et al. (12). It might be more beneficial to compare, in vivo, both "activated" and "non-activated" products that were similar in clotting factor activity.

FACTOR VIII BYPASSING ACTIVITY

In order to develop the most rational approach to finding an explanation for the bypassing activity of the prothrombin complex concentrates it is first necessary to correlate clinical and research observations with what is presently known about the mechanisms of coagulation and the in vivo, physiologic, control mechanisms known to prevent thrombotic episodes. The following information pertains to the particular characteristics of FEIBA or PCC's that are of major interest. To date, no experimental data has been

Table 5. Factor Concentrations of Autoplex®* and Immuno FEIBA®**.

	Factor Concentration (U/ml)			
	II	VII	IX	X
Autoplex				
1.+	7	128	45	4
2.	5	96	33	5
FEIBA				
1.	15	30	34	16
2.	14	44	34	17

* Hyland Laboratories
** Vienna, Austria
\+ Results of the determinations from two different lots

presented to relate FEIBA to a specific protein.

Elsinger (78) has fractionated FEIBA Immuno® on both Sephadex G-100 and dextran sulphate and demonstrated FEIBA in peaks from both columns but has not isolated or purified a specific protein or complex. Based on observations from Elsinger's work that FEIBA has a relatively high molecular weight on Sephadex G-100, Tishkoff and Hess (79) devised a number of experiments to try and demonstrate the existance of such a protein. Within the limits of the gel electrophoretic techniques used, they were unable to define a high molecular weight species having FEIBA. Barrowcliffe et al.(80) demonstrated the existance of a factor VIII-lipid complex in some PCC's and found that this complex was resistant to inhibition by the factor VIII antibodies. Although this could account for a minor portion of the bypassing activity the inconsistant amounts of factor VIII present do not correlate with the in vivo correction when

concentrates are injected nor the improved hemostatic function. Additional explanations for the bypassing activity that must be considered include the markedly elevated levels of coagulation zymogens following administration of PCC's and the presence of small amounts of activated clotting factors in these complexes.

Although it is extremely difficult to rule out contamination with activated clotting factors as the causitive agent in the bypass mechanism a number of observations would suggest only a minor contribution. First, the presence of factor VIIa in PCC's could provide a factor X activating mechanism in conjunction with the released tissue factor. Although this would account for some improved hemostasis at the site of tissue injury or bleeding, it does not explain the shortening of the NAPTT or APTT seen in the in vitro test system when PCC's are evaluated for FEIBA. Second, small quantities of both IIa and Xa are known to contaminate PCC's. These enzymes could potentially contribute to the thrombogenic character and/or to the occasional DIC that has been described in the literature. In vitro data (78), however, suggests that FEIBA is not readily inhibited by PMSF and recent work on the in vivo inhibition of Xa and IIa by AT III indicates a very rapid inhibition within the human circulatory system (81). Finally, IXa as well as being inhibitable by AT III requires as a cofactor the presence of IIa activated factor VIII. In the absence of the antihemophilic factor the rate of activation of factor X by

the IXa complex would be approximately 30,000 fold less than it is in the presence of factor VIIIa.

There are a number of articles in the literature documenting the existance of enzymatic activity in native zymogens (82-84). The most pertinent of these pertain to factor VII, chymotrypsinogen and trypsinogen. It has been shown by Nemerson et al. that the factor VII zymogen requires tissue factor for the activation of factor X. Because of the variable amounts of factor VII present in the PCC's and again because of the inability to explain the in vitro correction of the NAPTT or APTT via this mechanism the zymogen contribution by factor VII is questionable. It could be postulated that excessive amounts of either prothrombin or factor IX could contribute to FEIBA. Factor IX however, like factor IXa, would require as a cofactor the presence of factor VIII. Finally, based on the kinetics of the prothrombinase complex the concentration of II in plasma has been shown to be approximately 5x the experimentally determined K_m (85). This would indicate that although there is a large increase in the II concentration during PCC administration this zymogen increase would have little effect on the overall rate of the reaction. Therefore, because zero order kinetics already pertain, it is unlikely that increasing the II concentration would contribute significantly to enhanced hemostatic function.

Work by Tishkoff (86) has demonstrated that a major contributing factor to the bypassing activity is factor X.

They found that factor X in combination with trace amounts of IIa could partially correct the APTT of a factor VIII deficient plasma with a high titer inhibitor. It was thought that the major contribution of IIa was as an activator of factor V and that factor X appeared to be the major catalytic agent.

PROTHROMBINASE COMPLEX

If the factor VIII bypassing activity of the PCC's is in anyway related to what is presently known about the classic mechanisms of coagulation then the most likely enzyme system to be involved would be the prothrombinase complex (IIase). Because a major portion of the work to be presented involves an understanding of the complex interactions of the components of the IIase complex a review of the mechanism of prothrombin activation will be presented.

The components of the IIase complex include Ca^{+2}, phospholipid, factor Va and factor Xa. A few of the questions that need to be answered include: What is the role of Ca^{+2} and what is the mechanism of phospholipid, Xa and factor Va binding? Does the composition of the phospholipid make a difference? What is the source of phospholipid? How do phospholipid and Va effect the rate of II cleavage? Does binding of Ca^{+2} induce conformational changes in II and factor X or Xa? What are the K_m and V_{max} for the complete complex? And, what is the order of binding of the individual components?

Vitamin K

It has been established that vitamin K is essential for the normal production of several coagulant components. This critical vitamin K dependent, post-ribosomal, alteration results from the γ-carboxylation of specific glutamyl residues in factor II, VII, IX, X, protein C and protein S (87-90). The carboxylation requires a reduced vitamin K, O_2, CO_2 and a carboxylase complex which appears to include vitamin K epoxidase activity (91-93). A proposed mechanism suggested by Larson et al. is illustrated in Figure 1 (91). In addition the structure of glutamic acid and γ-carboxyglutamic acid (GLA) are shown in Figure 2 (94).

This vitamin K dependent modification of glutamic acid is necessary for calcium binding to occur and therefore for the physiologic activation of prothrombin and the lipid binding characteristics of factor II, IX, and X. In addition, the GLA residues impart to these proteins their unique ability to be adsorbed to barium salts (95-99). The importance of the GLA residues can be demonstrated in humans and animals when vitamin K antagonists such as dicoumoral are used as anticoagulants. Although the prothrombin molecule from either normal or dicoumoral treated individuals have identical amino acid compositions, the dicoumoral induced prothrombin will not bind Ca^{+2} and does not function normally (100-102). The defect has been shown to specifically involve the glutamic acid residues of the fragment 1 portion of the prothrombin molecule (103,104). The

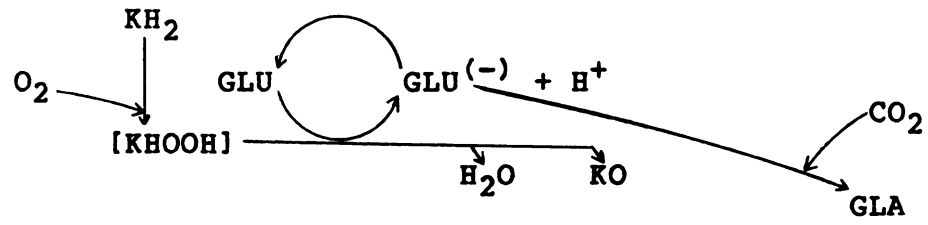
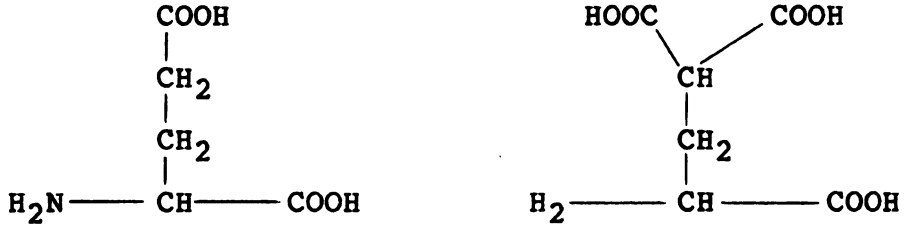


Figure 1. Carboxylation of Glutamic Acid.

identification of the GLA residues of the vitamin K dependent factors provided a specific amino acid that could be implicated in defining the mechanism whereby the components of the IIase complex associate to form a functional enzyme unit.



Glutamic Acid **γ-carboxyglutamic acid**

Figure 2. Structure of Glutamic Acid and γ-Carboxyglutamic Acid.

Calcium Binding and Protein Conformation

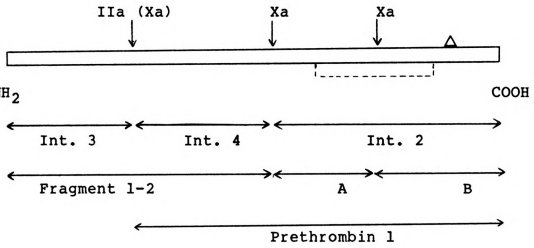
The majority of the work on the calcium binding characteristics of the vitamin K dependent proteins has been done using prothrombin as a model. However, there are many parallels with factor X as well as the additional coagulant proteins.

When analyzed by SDS electrophoresis human prothrombin has a molecular wight of approximately 72,000. The amino acid sequence of both human and bovine prothrombin are very

similar and there is extensive sequence homology among the proteinases of blood coagulation particularly as noted by the conservation of the charge relay system of the serine proteases and the nearby residues in the 3-dimensional structure (105). The activation intermediates of human prothrombin have been determined and are illustrated diagramatically in Figure 3. The Xa and IIa cleavage sites are included as well as alternate designations for the prothrombin fragments (106,107).

As will be seen, an understanding of the various activation fragments of the prothrombin molecule will facilitate an understanding of the studies on Ca^{+2} binding, Va binding and conformational changes induced by Ca^{+2} and other divalent cations.

As we have seen the functional characteristics of the vitamin K dependent proteins as either substrates or ultimately as proteolytic enzymes depends on their Ca^{+2} binding characteristics and subsequently their ability to bind a phospholipid surface. Papahadjopoulus and Hanahan were the first to demonstrate that Ca^{+2} ions were necessary for the physical integrity of the IIase complex and that while they were not important for the binding of factor Va they were essential for the Xa mediated cleavage of the prothrombin molecule (108). The calcium binding characteristics of prothrombin were more easily studied than those of the other vitamin K dependent proteins for two reasons: 1) prothrombin is present in large quantities and



Fragment 1	Int. 3	23,000
Fragment 2	Int. 4	13,000
Prethrmobin 1	Int. 1	51,000
Prethrombin 2	Int. 2	41,000
Fragment 1-2	Int. 3-4	36,000

Figure 3. Prothrombin Activation Fragments. Prethrombin 2 or Int. 2 is the immediate precursor of α-thrombin which has A and B chains linked by a disulfide bond.

is easily isolated and 2) unlike the other vitamin K dependent proteins the γ-carboxyglutamic acid region of prothrombin is not linked to the rest of the molecule by disulfide bonds. Therefore, isolation of the Ca^{+2} binding region is facilitated. As previoulsy noted the fragment 1 or intermediate 3 portion of the prothrombin molecule has been shown to contain all of the Ca^{+2} binding sites (109-111). The binding of Ca^{+2} to fragment 1 has been studied using a variety of methods including equilibrium dialysis, Hummel and Dreyer gel filtration, steady state dialysis and paramagnetic relaxation rates (112-117). A number of observations can be made based on these techniques. First,

there are approximately 6-10 calcium binding sites per prothrombin molecule with a dissociation constant of 6.3×10^{-4}. Second, because of the relatively weak binding of Ca^{+2} to the prothrombin molecule studies can only be done at fairly high protein concentrations and greater effects from protein dimerization can be expected. In contrast, because of the relatively tight binding of manganese ($K_d = 2.2 \times 10^{-5}$) and gadolinium ($k_d = 1.6 \times 10^{-7}$) equilibrium studies can be done at much lower protein concentrations. This facilitates evaluation of the binding characteristics and provides a model to which the Ca^{+2} binding can be compared. Studies using trivalent lanthanide ions Mn(II) and Ca(II) have demonstrated two separate classes of binding sites for both factor X and prothrombin (112, 114, 118-120). In each case, two high affinity sites were identified and a number of low affinity sites were found. Finally, in addition to determining the number of metal binding sites and the affinity of those sites, most of the observations on the metal-binding characteristics have evidenced complex binding. Both positive cooperativety, a process similar to that observed with the O_2 saturation curve of hemoglobin, and negative cooperativety, a process reflecting multiple classes of binding sites. If a process reflecting positive cooperativety is taking place then some mechanism, other than simple Ca^{+2} binding, must be looked for. Prendergast and Mann and Jackson, et al. observed a cation mediated self association of prothrombin fragment 1 in the presence of Ca^{+2} (116,117). This dimerization phenomenon may be of no

significance as protein concentrations that reflect physiologic levels fail to demonstrate complex binding characteristics and only when protein concentrations are much higher is positive cooperativity demonstrated. Alternatively, a change in the stoichiometry of the factor X or prothrombin molecule could account for an observation consistent with positive cooperativity. It has been observed that binding of several di and trivalent cations induces a spectral shift and flourescent quenching of specific tryptophan residues resulting from a conformational change in the molecule (116,121,122). Lewis et al. and Keyt et al. have also isolated Ca^{+2} induced conformation specific antibodies to both prothrombin and factor X (123,124). Based on the flourescent quenching, immunochemical information, the presence of 2 high affinity Ca^{+2} binding sites and circular dichroic studies by Bloom and Mann (125) there is adequate data to support positive cooperation between the two high affinity Ca^{+2} binding sites resulting in an alteration in the tertiary structure of prothrombin or Factor X (126). Nelsestuen has shown that prothrombin must undergo the Ca^{+2} dependant conformational change prior to binding lipid and that an intact secondary and tertiary protein structure is required to form the phospholipid binding region (127). Physiologically, it is possible that prothrombin could circulate with the high affinity Ca^{+2} sites bound. This statement is based on the fact that half-maximal perturbation of the prothrombin molecule is seen at

a Ca^{+2} concentration of 0.22 mM (118) and physiologic levels of Ca^{+2} are in the range of 1 mM.

Factor V

Factor V is a high molecular weight glycoprotein that has been purified to homogeniety by several investigators (128-131). The single chain factor V, prior to involvment in the prothrombinase complex, is activated by human IIa or the factor V activator from Russells viper venom (RVV-V) to Va (132-134). Suzuke et al. demonstrated four fragments upon thrombin-catalyzed activation consisting of fragment D (105,000), fragment E (71,000), fragment Cl (150,000) and a dimer fragment 1-2 (71-74,000) (134). The biologically active fragments appeared to be fragment D noncovalently bound to fragment 1-2. In addition to activating factor V, thrombin will eventually proteolytically degrade the Va activity (126).

Factor Va accelerates the rate of prothrombin activation. Nesheim et al. showed a 13,000 fold rate enhancement of the prothrombinase complex when the complete complex was compared to a Xa-Ca^{+2}-phospholipid complex and a 278,000 fold enhancement of the complete complex over that of the enzyme, Xa alone as determined in an amidolytic assay (85). According to Rosing et al. the effect of Va is mainly to increase the V_{max} as opposed to lipid which has its major effect on decreasing the K_m (136). The binding of factor Va to prothrombin appears to be through the fragment 2 region of the prothrombin molecule and although there appears to be a single Ca^{+2} binding site that is necessary

for Va activity, the binding of factor Va to lipid is a Ca^{+2} independent process (137,138).

Phospholipid: Surface Assembly

In vivo, the classically defined mechanism for the assembly of the prothrombinase complex is via interaction with platelet surfaces, in particular platelet factor 3. Miletich et al. have demonstrated that factor V(Va) is responsible for the binding of factor Xa to the platelet surface (139,140). In addition to the Va receptor, phospholipid has been proposed to be involved although the exact mechanisms are unknown.

Studies evaluating the total effect of the prothrombinase complex on the II$\longrightarrow$IIa conversion have substituted phospholipid monolayers for platelet membranes. It has been reported that the platelet surfaces catalyze prothrombin activation 15x the rate of lipid monolayers. Nesheim et al. found, however, that under their experimental conditions there was no difference in the catalytic efficiencies of a system containing platelet lipid and Va and one containing added phospholipid and Va (85). They also concluded, as did Militich et al. (139) that factor Va comprises the Xa binding site on the phospholipid surface as well as on platelets.

Both prothrombin and factor X bind phospholipid surfaces and a considerable amount of work has gone into defining a model lipid system (126,127,141). The main feature appears to

be the need for an acidic phospholipid, of which phosphotidylserine is the most effective (142,143). The binding, as noted earlier, is Ca^{+2} dependent and requires a metal induced protein transition in prothrombin and factor X. Work by Lim et al. indicated that prothrombin binds via the tip of fragment 1 and extends radially from the membrane surface (144). Factor X also appears to bind at one end and extend into solution. Mayer et al. using surface pressure measurements concluded that binding of prothrombin resulted in only minor surface pertubations and therefore, there was not significant insertion of the protein into the monolayers (145). In addition to the platelet or model lipid source providing a means by which the prothrombinase complex is assembled, it may also provide a means of concentrating the components of the enzyme complex and increasing their local concentration.

It is interesting to note that although lipid model studies have given a good indication about the type of interaction necessary to assemble the prothrombinase complex, the specific type of in vivo reactions are not clear. Nemerson and Furie speculated that it would be possible for any mechanism capable of binding Ca^{+2} and organizing the protein array to optimize coagulation (135). Therefore, platelet lipid does not necessarily provide the appropriate surface.

Although the specific mechanism of assembly of this complex and the order in which it occurs is unkown, based on the data available one can postulate on possible mechanisms.

Kosow and Orthner, after a kinetic evaluation of the activation of human prothrombinase by Xa, Ca^{+2} and phospholipid, showed that phospholipid appeared to be the second reactant to bind to the enzyme (146). This is consistent with a mechanism in which two high affinity binding sites undergo a Ca^{+2} dependent cooperative binding process that results in a conformational change in factor X and II. This change, which is necessary for membrane binding, results in the exposure of, in the case of prothrombin, 6 additional Ca^{+2} binding sites and, in the case of factor X, as many as 16-20 Ca^{+2} binding sites, which are composed of γ-carboxyglutamic acid residues and utilize the binding of Ca^{+2} to bridge the negative charged lipid surface to the negative charge of the GLA residues. Whether prothrombin or factor X(Xa) bind first is not known. The site on the lipid surface for binding Ca^{+2} would be the phosphate group but the specific platelet receptor has not been well defined. Along this line, it has been demonstrated that minimal prothrombinase activity exists with inactivated platelets but upon activation procoagulant activity increases significantly because of the internal location of the negatively charged phospholipids (147). Finally, factor Va appears to serve as the binding site for the X(Xa) molecule and to increase the maximum velocity of the reaction. Whether or not this alters the proteolytic activity of X(Xa) or provides a more favorable steric arrangement or both is not certain.

MATERIALS AND METHODS

Chromatography

Deae-Sephadex A-50, Sepharose 4B, QAE-Sephadex A-50 and Sephadex G-25 were the products of Pharmacia, Piscataway, N.J. Ultrogel AcA-34 was purchased form LKB Ltd. Rockville, MD. Heparin-Sepharose (148), benzamindine-Sepharose (148), poly(homoarginine)-Sepharose (148), RVV-X-Sepharose (106) and AT III-Sepharose were prepared by coupling the ligand to Sepharose-4B activated with cyanogen bromide.

Proteins

Protein preparations were assessed for homogeneity by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (149). The purity of factor V was determined on 5% gels and the standards used in both cases were carbonic anhydrase (M.W. 29,000), egg albumin (M.W. 45,000), bovine ablumin (M.W. 66,000), phosphorylase B (M.W. 97,400), β-glactosidase (M.W. 116,000) and myosin (M.W. 205,000). Protein samples were evaluated in both a reducing, 1% v/v 2-mercaptoethonal, and non-reducing system. Protein concentrations were estimated from the absorbance at 280 nm

using the following values for $E_{280\ nm}$: factor X, 1.16; prothrombin, 1.38 (148); factor Xa, 0.96 (150); factor V, 0.89 (128); and AT III, 0.61 ml $mg^{-1}cm^{-1}$ (151). The partially purified protein concentration of factor VII was estimated using the equation of Kalachar where protien concentration in mg/ml = $1.45D_{280} - 0.7D_{260}$ where D_{280} and D_{260} are optical densities at 280 nm and 260 nm respectively (152).

Factors II, X and IX Purification

Factors II, X and IX were purified as described by DiScipio et al. All procedures, unless otherwise indicated, were performed at 4°C (148).

Five liters of cryo-poor plasma anticoagulated with CPD-A (Great Lakes Regional Red Cross Blood Services, Lansing, MI.) were pooled in a large plastic container. Heparin (50,000 units), 1.5 mM O-phenanthroline and 250 mg of polybrene were added and the plasma was stirred for 15 minutes. Two hundred ml of 1M barium chloride was then added with continuous stirring over a period of 30 minutes and the stirring was continued for an additional 15 minutes. The plasma was centrifuged at 8,000 xg for 15 minutes and the supernatant was discarded. The barium citrate pellet was then washed twice with 1 liter each of 0.02 M imidazole buffer, pH 6.0, containing 0.2 M NaCl and 1 mM benzamidine.

The adsorbed protein was eluted from the barium citrate pellet by homogenization in a siliconized Waring blendor with 1 liter of 0.2 M Mes-HCl, pH 5.85, conatinig 0.15 M sodium citrate, 10 mM benzamidine, 1000 units of heparin and

10 mg of soybean trypsin inhibitor (STI). The mixture was then stirred for 1 hour and the precipitate was removed by centrifugation at 8,000 xg for 20 minutes.

The supernatant from the barium citrate eluate was brought to 10% saturation with solid, enzyme grade, ammonium sulfate by addition of 76 g of finely ground ammonium sulfate containing 1.5 ml of 1 M Tris base as a buffering agent. After addition of the ammonium sulfate the solution was allowed to stir for an additional 15 minutes. The precipitate was removed by centrifugation at 8,000 xg for 15 minutes and discarded. The supernatant was brought to 40% saturation by the slow addition of 186 g of finely ground ammounium sulfate containing 9 ml of 1 M Tris base. The precipitate was again removed and discarded and the supernatant was brought to 70% saturation with 229 g of ammonium sulfate containing 10 ml of 1 M Tris base. The precipitate from this cut was collected by centrifugation at 8,000 xg for 30 minutes and then dissolved in 50 ml of 0.02 M Tris-H_3PO_4, pH 5.85, containing 0.1 M EDTA, 2 mM benzamidine, and 0.02% sodium azide. This solution was then dialyzed overnight against 1 liter of the same buffer followed by two, one liter changes of 0.02 M Tris-H_3PO_4, pH 5.85, containing 2 mM benzamidine and 0.02% sodium azide. A fine precipitate developed during dialysis and was removed by centrifugation at 9,000 xg for 10 minutes.

The protein was applied to a DEAE-Sephadex-A50 column (2.6 x 36 cm) equilibrated with 0.02 M Tris-H_3PO_4, pH 5.85,

2 mM benzamidine and 0.02% sodium azide. The column was washed with 250 ml of the same buffer plus 0.15 M NaCl and eluted with a gradient consisting of 250 ml of 0.02 M Tris-H_3PO_4, pH 5.85, 2 mM benzamidine, 0.02% sodium azide and 0.15 M NaCl and 250 ml of the same buffer with 0.55 M NaCl. The column flow rate was 66 ml/hr and 3.5 ml fractions were collected. The peak of protein containing the factor X, II, and IX activity was pooled and dialyzed against 3 one liter changes of 0.02 M Mes-Tris, pH 5.9, 2 mM benzamidine and 0.02% sodium azide.

The protein obtained from the DEAE column was then applied to a benzamidine-Sepharose column (1.6 x 30 cm) equilbrated with 0.02 M Mes-Tris, pH 5.9, 2mM benzamidine, 0.02% sodium azide. The column was washed with the same buffer containing 0.3 M NaCl and then eluted with a linear gradient consisting of 250 ml of the above buffer with 0.3 M NaCl and 250 ml with 1.3 M NaCl. The flow rate was 24 ml/hr and 3.5 ml fractions were collected. The factor IX and X activity were seperated on this column.

The factor X activity was pooled and immediately brought to 1 mM PMSF and applied to a poly(homoarginine)-Sepharose column (1.6 x 30 cm) equilibrated in 0.02 M Mes-Tris, pH 5.9, 0.02% sodium azide. The column was washed with 250 ml of 0.02 M Mes-Tris, pH 5.9, 0.02% sodium azide, 3.0 M NaCl and 2×10^{-4} M PMSF. The protein was then eluted with 250 ml of Mes-Tris buffer containing 2.4 M NaCl and 2.9 M quanidine hydrochloride and 1 mM PMSF. The column was run at 16 ml/hr and 2.5 ml fractions were collected.

The factor X from this column was pooled and brought to 10 mM in benzamidine and the dialyzed against 1 liter of Mes-Tris buffer containing 2.4 M NaCl and 10 mM benzamidine. Following this the factor X was then dialyzed against two 1 liter changes of 0.02 M Mes-Tris, pH 5.9, 2 mM benzamidine and 0.02% sodium azide. The factor X was stored at -80°C.

The factor IX activity from the benzamidine-Sepharose column was pooled and concentrated to approximately 60 ml on an Amicon PM-10 membrane. The concentrate was then dialyzed overnight against 1 liter of 20 mM Mes-Tris, pH 5.9, 2 mM benzamidine and 0.02% sodium azide. Following this the sample was dialyzed an additional 8 hours against the same buffer containing 50 mM NaCl. At the end of this dialysis $CaCl_2$ was added to a final concentration of 2.5 mM and the sample was brought to 1mM with 0.1 M PMSF. The sample was applied to a heparin-Sepharose column (1.6 x 13 cm) that had previously been layered with 2 cm of Sepharose-4B and equilibrated with 20 mM Mes-Tris, pH 5.9, 2 mM benzamidine, 2.5 mM $CaCl_2$, 0.02% sodium azide and 50 mM NaCl. The flow rate was maintained at 20 ml/hr and 2.5 ml fractions were collected. After the sample application the column was washed with 60 ml of equilibrating buffer. The protein was then eluted with a linear gradient consisting of 100 ml of equilibrating buffer containing 1 mM PMSF and 100 ml of the same Mes-Tris buffer containing 1.5 M NaCl and 1 mM PMSF. Following elution of prothrombin the gradient was stopped and the column was washed with 100 to

150 ml of Mes-Tris buffer containing 0.3 M NaCl and 1 mM PMSF. The gradient was then reapplied and the factor IX activity eluted. The peak of prothrombin activity and factor IX activity were pooled seperatly, brought to 5.0 mM EDTA and dialyzed 5 hours against 1 liter of 20 mM Mes-Tris, pH 5.9, 2 mM benzamidine, 0.02% sodium azide and 50 mM EDTA and then overnight against 20 mM Mes-Tris, pH 5.9, 2 mM benzamidine, 0.02% sodium azide and 0.1 M NaCl. The samples were concentrated on a Amicon PM-10 membrane and stored at $-80^{o}C$.

Factor VII Purification

In the DiScipio procedure, factor VII eluted on the back half of the leading protein peak from the DEAE-Sephadex-A50 column. The factor VII activity was pooled and brought to 10 mM in benzamidine and further purified as follows:

The purification of factor VII was a modification of the procedure of Broze and Majerus (153). The pooled factor VII activity, obtained as previously described, was dialyzed for 8 hours against two 1 liter changes of 0.05 M Tris HCl, pH 8.0, 0.15 M NaCl and 10 mM benzamidine. The sample was then applied to a QAE-Sephadex column (1.6 x 30 cm) equilibrated in the same buffer. The flow rate was maintained at 20 ml/hr and 2 ml fractions were collected. The column was then washed with 100 ml of equilibrating buffer and the factor VII was eluted with a buffer containing 50 mM Tris-HCl, pH 8.0, 0.135 M NaCl, 0.005 M $CaCl_2$ and 10 mM benzamidine. Each fraction collected

contained 50 ul of 0.5 M Na_2EDTA, 2.0 M Tris-HCl, pH 8.0. The fractions containing factor VII activity were immediately pooled and concentrated to approximately 4 ml. The concentrate was dialyzed overnight against 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl and 10 mM benzamidine. The factor VII activity was stored at -80°C and prior to use was desalted on Sephadex G-25 to remove the benzamidine.

Factor Xa

Human factor Xa was prepared by the method of Downing et al. (106) using the factor X activating enzyme from Russell's viper venom (RVV-X) prepared according to the procedure of Kisiel et al (154). The following modifications were made. Sephadex G-100 was substituted for Sephadex G-150 and all isolation procedures were preformed at 4°C. After fractionation on QAE-Sephadex the fractions having RVV-X activity were pooled and run on a Sephadex G-25 column equilibrated in 0.5 M NH_4HCO_3 and immediately lypholized. The lypholized samples were stored at -20°C.

Factor X zymogen was activated by incubation with RVV-X-Sepharose in the presence of 6 mM $CaCl_2$ for 10 min at 37°C as follows:

Sepharose-4B was activated in the conventional manner. After activation the gel was washed with 0.0168 M imidazole, pH 7.4, 0.145 M NaCl and then mixed with a volume of buffer equal to the packed gel volume containing 10 mg/ml of purified RVV-X (154). After gentle stirring at 4°C overnight

the buffer-protein mixture was removed and the same volume of 1 M ethanolamine, pH 8.0, was added and stirring continued for an additional 2 hours at room temperature. The gel was then washed alternately with 0.1 M bicarbonate buffer, pH 9.5, 1 M NaCl and 0.1 M acetate buffer, pH 4.0, 1 M NaCl. The gel was then equilibrated with 0.0168 M imidazole, pH 7.4, 0.15 M NaCl. Prior to activation factor X was dialyzed against 0.0168 M imidazole, pH 7.4, 0.15 M NaCl. Both the factor X and the RVV-X-Sepharose were brought to a final concentration of 6 mM $CaCl_2$ and approximately equal volumes of RVV-X-Sepharose and factor X were combined and incubated, with mixing, at 37°C. The reaction was stopped by addition of 0.2 M tetrasodium EDTA to a final concentration of 20 mM when the Xa activity reached a maximum. The Xa was then dialyzed against 20 mM Mes-Tris, pH 5.9, 2 mM benzamidine and 0.02% sodium azide and stored at -80°C. Prior to use the benzamidine was removed by dilution to give a final concentration of less than 4×10^{-6} M in the assay mixture. SDS-PAGE of factor Xa indicated that the preparation was homogenous. The active site concentration of factor Xa was determined by titration with p-nitrophenyl-p'-guanidinobenzoate (p-NPGB) according to the procedure of Smith employing an experimentally determined molar extinction coefficient for p-nitrophenol of $E_{402nm} = 15{,}317\ M^{-1}cm^{-1}$ (155).

Factor V

Factor V was isolated according to the method of Kane and Majerus (128). The following modifications were made: Fresh plasma was substituted for fresh frozen plasma as there appeared to be some activation products resulting from the use of the latter. In addition, PMSF was used in place of DFP as the active fluoride inhibitor. PMSF, was added immediately prior to the use of any buffer. The final product had a specific activity of 75 U/mg and showed a single molecular weight band on SDS-PAGE.

Activation of factor V was carried out in the following manner. The protein was desalted on an AcA-34 column equilibrated with 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl and 5 mM $CaCl_2$ to remove the benzamidine. Factor V (200 ug/ml) was incubated with α-thrombin (2 U/ml) for 5 min at 37°C in the same buffer. Thrombin was removed by adsorption onto benzamidine-Sepharose followed by filtration on a mini-column to remove the gel.

Antithrombin III and Fibrinogen

Purified AT III was generously provided by Dr. M. Wickerhauser (American Red Cross, Bethesda, MD.) and contained 8.1 mg/ml of AT III and was 95% pure by SDS-PAGE.

Human fibrinogen was obtained from Kabi Group, Inc., Greenwich, CT. A stock solution was prepared by dissolving 1.5 g in 66 ml of distilled water and then dialyzing for 12 hours against 2.4 mM sodium citrate-50 mM sodium acetate-

0.15 M NaCl, pH 8.2. The protein was stored at -80°C.

Phospholipids

Phospholipid vesicles were prepared according to the procedure of Kosow and Orthner (146), and Nelsestuen and Lim (144). A chloroform suspension, prepared by mixing 50 mg of L-α-phosphatidylethanolamine and 22 mg of Folch Fraction III (Sigma Chemical Co., St. Louis, MO.), was used routinely as a source of phopholipid. After sonication and centrifugation, the phospholipid suspension was chromatographed on a Sepharose-4B column proviously primed with isotonic saline extract of rabbit brain followed by thorough washing and equilibration with 20 mM triethanolamine, pH 7.4. The molar concentration of lipid in the stock solution was determined by the method of Gomori (156). Dilutions of the stock solution were made in 50 mM triethanolamine, pH 7.4, 0.2 M NaCl.

Addtitional Reagents

Morpholinoethanesulfonic acid (Mes), Trizma base, quanidine hydrochloride, benzamidine, heparin sodium salt (grade 1, 164 units/mg), poly-L-lysine (M.W. 150,000-300,000), p-aminobenzamidine hydrochloride, ε-amino-N-caproic acid, 1-cyclohexyl-3-(2-morpholinoethyl-carbodiimide, Ressull's viper venom, polyethylene glycol 6000 and p-nitrophenol were purchased from Sigma Chemical Company, St. Louis, MO. Triethanolamine hydrochloride, O-methylisourea hydrogen sulfate and cyanogen bromide were obtained from Aldrich Chemical Company, Milwaukee, WI.

p-NPGB was purchased from ICN Biochemicals, Cleveland, OH. All other reagents were of the highest purity available. p-(Amidinophenyl)methanesulfonyl flouride (p-APMSF) was a generous gift of Dr. David Bing, Boston, MA (157).

Clotting Factor and FEIBA Assay Procedures

Prothrombin, factors X, Xa, and IX were assayed by methods proviously described (158). Factors V and Va were assayed by a modification of the one-stage procedure of Quick (159) where one unit of activity is defined as the amount of activity present in 1 ml of pooled normal plasma. Factor V deficient plasma, ACTIN®, Factor IX deficient plasma and Activated Thromboplastin were obtained from American Dade, Miami, FL.

FEIBA was monitored by the clot promoting activity of the test protein in the activated partial thrmoboplastin time (APTT) or nonactivated partial thromboplastin time (NAPTT) assay employing either factor VIII deficient plasma or factor VIII deficient plasma with an inhibitor as substrate. Inhibitor plasma was provided by Dr. John Penner, Michgian State University, East Lansing, MI. Individual APTT assays were performed as follows: The test sample (0.1 ml) and 0.1 ml of factor VIII deficient or inhibitor plasma were mixed together and warmed at 37^{o}C for exactly 1 minute. ACTIN® (0.1ml) was added and the incubation was continued for an additional 2 minutes. Recalcification was accomplished by the addition of 0.1 ml of prewarmed 0.02 M $CaCl_2$ and the clotting time recorded.

The results are expressed in seconds as the average of duplicate determinations. NAPTT assays were performed as for the APTT except rabbit brain cephalin (Sigma Chemical Co., St. Louis, MO.) was substituted for ACTIN®.

Prothrombin Activation Assay

The assay system used was a modification of the procedure of Kosow and Orthner (146) where prothrombin activation in a steady state system is monitored by the generation of thrombin; thrombin peptidase activity was assayed on the peptide anilide S-2238 (H-D-Phe-Pip-Arg-p-nitroanilide HCl, Ortho Diagnostics, Raritan, N.J.). The activation of prothrombin was carried out in disposable semimicro cuvettes at 30°C. The reaction mixture, in a total volume of 1 ml, consisted of variable concentrations of factor X (or factor Xa), prothrombin and phospholipid as specified for individual experiments under "Results". In addition, the reaction mixture contained 1 U/ml of factor Va, 2.5 mmol/ml $CaCl_2$ and 100 umol/ml of S-2238 in 50 mM triethanolamine, pH 7.4, 0.16 M NaCl buffer. The assay was performed as follows: Factor X or Xa, factor Va, $CaCl_2$ and phopholipid were preincubated for 2 minutes. At the end of this time S-2238 was added and the endogenous rate of activation was followed for 1 min. The reaction was initiated by the addition of prothrombin. The rate of release of p-nitroaniline was monitored by a DU-spectrophotometer at 410 nm with Gilford adaptations and a chart

recorder. The spectrophotometric data were fitted to the equation $A_{410\ nm} = at^2/2$ according to the procedure of Kosow and Orthner (146). The slope of this line, which is directly proportional to the rate of prothrombin activation, is converted to the concentration of thrombin formed per unit time using a molar extinction coefficient for p-nitroaniline ester of 9400 and an experimemtally determined first order rate constant of k = 55/s for the cleavage of S-2238 by α-thrombin. This rate constant is in very close agreement with that determined by Kosow and Orthner (146). The endogenous rate due to spontaneous cleavage of the chromogenic substrate was subtracted from the absorbance readings prior to determination of the rate of prothrombin activation (See Appendix).

RESULTS

Protein Isolation and Characterization

Prothrombin Complex Proteins

Factors II, IX and X were purified to homogeneity from cryoprecipitated fresh frozen human plasma. The criteria for determining homogeneity consisted of the presence of a single band of protein on 7.5% SDS-gels, the absence of any detectable contamination with other clotting factors of the prothrombin complex and, in the case of factor X, amino terminal sequencing of the purified protein. Prothrombin and factor IX evidenced a single band on both reduced and non-reduced SDS-gels and factor X was a single band in a non-reduced system and demonstrated a heavy and light chain in a reducing system (Figure 4). The molecular weight of the purified prothrombin molecule was 72,000, factor IX was 58,000 and Factor X had a heavy chain molecular weight of 49,000 and a light chain molecular weight of 17,000. The only modification in the DiScipio procedure was the use of PMSF in the final stages of the factor X preparation and during heparin affinity chromatography. There was no detectable Xa activity in any of the isolation procedures (<0.001U/ml when determined by comparison to a Xa standard).

It was necessary to carefully monitor the elution of factor X and IX from the benzamidine-Sepharose column. With multiple use, there was a decrease in the affinity of the benzamidine-sepharose for the proteins. As a result, adjustments in the salt gradient to lower ionic strengths was required to maintain adequate seperation of factor IX and X.

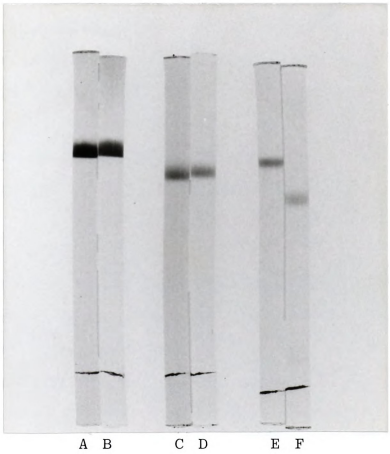


Figure 4. Prothrombin complex proteins isolated and purified by the procedure of DiScipio. Proteins samples were run on 7.5% SDS-polyacrylamide gels in both a reducing buffer (2-mercaptoethanol) and a non-reducing buffer. A and B, prothrombin reduced and non-reduced; C and D, factor IX reduced and non-reduced; E and F, factor X reduced and non-reduced.

Factor V

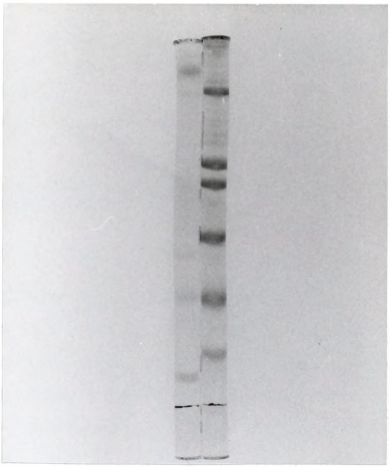
Factor V was isolated from fresh plasma as apposed to fresh frozen plasma. This change was made because of problems that arose with a precipitate in cryoprecipitated plasma that did not redissolve on thawing at 37°C. It is possible that limited activation of the plasma occured with the resulting formation of fibrin. The use of fresh frozen plasma also resulted in the coelution of several different contaminating bands along with the factor V. When fresh plasma was used, factor V was isolated as a single band of protein with a molecular weight on 5% SDS-gels of approximately 300,000 (Figure 5). The specific acitivity was approximately 70 U/mg and could be activated 10-15 fold upon incubation with 2 U/ml of thrombin.

Factor VII

Factor VII was isolated as a partially purified protein by the modifications described in the methods section. With these modifications we obtained a 65,000 fold purification with a specific acitivity of approximately 900 U/mg. Although the factor VII was not purified to homogeniety there was no contamination with other components of the prothrombin complex, as determined by specfic factor assays, and it was therefore deemed suitable for in vitro coagulation studies that examined the effect of factor VII on FEIBA.

Amino Terminal Sequencing

As part of the factor X characterization the Protein Sequencing Laboratory of the University of California, Davis, performed amino-terminal sequencing on the purified factor X. We were concerned about two particular issues.



A B

Figure 5. Purified factor V as determined by 5% SDS-PAGE (A). The molecular weight standard (B) is included for comparison and consists of the proteins described in the Materials and Methods section.

One, we wanted the factor X protein characterized to the point that there was no question about the purity and identity of the molecule when compared to what had been

previously described in the literature. Two, because of the unusual characteristics we are ascribing to the factor X molecule we felt it was necessary to be sure that there was no alteration in the amino-terminal sequence that would be indicative of proteolytic alterations. The results obtained are shown in Table 6.

Table 6. Amino Terminal Sequence of Factor X

Heavy Chain	-	Ser	Val	Ala	Gln	Ala	Thr	Ser
Light Chain	-	Ala	Asn	Ser	Phe	Leu	Glu	Met

In Vitro Characterization of the Factor Eight Inhibitor Bypassing Acitivity

FEIBA Assay

In order to determine the in vitro effectiveness of factor X in a clotting system the FEIBA assay was used. We performed the initial assays after both one and forty minute preincubations of factor X or Xa with either the factor VIII inhibitor plasma or factor VIII deficient plasma without an inhibitor. The rationale for these incubation times is based on the assumption that after a forty minute preincubation endogenous inhibitors (AT III) would have destroyed activity due to contamination with or, in some experiments, intentional addition of active enzyme components. Figure 6 graphically illustrates the coagulation results using the FEIBA assay with factor X as the test sample and factor Xa as the control. As can be seen, either

protein will correct the prolonged clotting time at one minute. However, after forty minutes of incubation the Xa activity has been eliminated and only the factor X zymogen evidences any ability to still correct the prolonged clotting time. The ability to correct the prolonged clotting time was seen in both the APTT and the NAPTT.

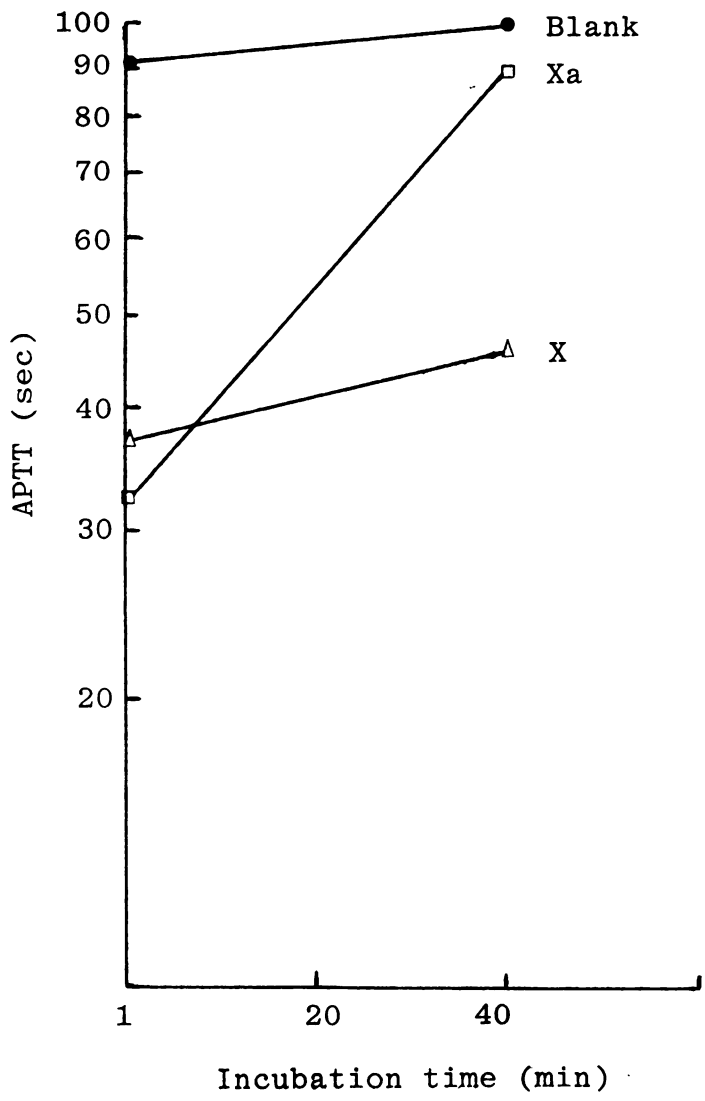


Figure 6. Corrective effect of factor Xa and factor X on APTT of factor VIII deficient inhibitor plasma. The assay contained 0.05 units of Xa or 0.5 units of X, expressed in clotting units.

Effect of AT III on FEIBA

In order to varify the assumption that endogenous AT III was in fact inhibiting Xa and, in addition, to eliminate any contamination of factor X with factor Xa, both factor X and Xa were preincubated with an excess of purified AT III. Experiments were conducted in two ways. 1) Factor X or Xa were incubated with an aliquot of AT III and 0.1 ml of this mixture was transferred to the FEIBA assay to determine if any bypassing activity remained when compared to controls that had been treated in an identical manner but without AT III. 2) Factor X or Xa was incubated with an equal amount (v/v) of AT III-Sepharose. The Sepharose was removed on a small column and the eluate was assayed for activity. In this case the controls were incubated with an equal amount of Sepharose that had been prepared without addition of AT III. This protocol was followed in order to determine if adding an excess of AT III had any effect on components, other than Xa, that are necessary for clot promotion in the FEIBA assay. The results of both approaches were essentially identical. Factor Xa activity was almost completly destroyed whereas the X activity was only slightly altered (Table 7). The small change in activity seen upon incubation of factor X with AT III was most likely due to inhibition of trace amounts of the active enzyme component, factor Xa.

Table 7. Effect of AT III-Sepharose on Factor X and Factor Xa FEIBA

Reaction Mixture	APTT (sec)	NAPTT (sec)
Buffer Blank	110	560
Factor X	68	151
Factor X + AT III	74	196
Factor Xa	13	23
Factor Xa + AT III	72	114

Experiments were performed in the presence of equal volumes of AT III-Sepharose (9 mg protein/ml) and either factor Xa or factor X zymogen. The reactants were incubated at 25°C for 60 min and the AT III-Sepharose removed by filtration on a mini-column (1.4 x 6.5 cm). Following removal of AT III-Sepharose, the filtrate was assayed for FEIBA using factor VIII inhibitor plasma (45 Bethesda units) as substrate. The assay contained factor Xa (8.6 nmols) or factor X (0.27 umols). A control reaction mixture consisted of Sepharose that had been activated and treated in a manner identical to the AT III-Sepharose but without the addition of the ligand.

Effect of p-APMSF on FEIBA

(p-Amidinophenyl)methanesulfonyl fluoride has been shown to be a specific, irreversible inhibitor of serine proteases. It has a specificity for the postively charged side chains of lysine or arginine. It has also been shown to react with the diisopropylfluorophosphate (DFP) reactive site (157). We were able to demonstratate a complete loss of Xa activity in the FEIBA assay as a result of preincubation of Xa with a 600 M excess of p-APMSF. Although Xa activity was lost using the same experimental conditions there was little or no change in the Factor X coagulant activity (Table 8). Several problems exist, however, that could adversly affect the results of these experiments. First, it is possible that there might be a difference in the rate of inhibition of

purified factor Xa and the small amount of Xa that would theoretically be contaminiating the factor X product. Second, the half-life of p-APMSF is sufficiently short so that concern about the final concentration after a 1 hour incubation was necessary. Finally, autocalalytic activation of the X molecule was a major concern during prolonged dialysis to remove excess inhibitor. For these reasons the following modifications were made in the experiment. In order to eliminate the effect of large variations in protein concentrations similiar molar concentrations of Factor X and Xa were used in the incubation mixture. Next, the concentration of p-APMSF was raised to 1 mM and an additional 1 mM was added after 30 minutes of incubation in order to counter any loss in activity due to the short half-life. The total incubation period was maintained at 1 hour. Finally, in order to minimize autocalalytic activation, the inhibitor was removed on a Sephadex G-25 column and the factor X or Xa was assayed immediately for any change in activity. The results of these procedural modifications had little, if any, effect on the outcome of the FEIBA assay.

Effect of Prothrombin Complex Proteins on FEIBA

In order to determine if Factor II, VII or IX had FEIBA, assays were run at varying concentrations of these proteins. Each protein was tested individually and in combination with Factor X. Assays were performed according to the following protocol. 1) Both one minute and 40 minute

FEIBA assays were run on individual factors. 2) Both one minute and 40 minute FEIBA assays were performed on various

Table 8. Effect of p-APMSF on Factor X and Factor Xa FEIBA

Reaction Mixture	APTT (sec)	NAPTT (sec)
Buffer Blank	104	462
Factor X	57	122
Factor X + p-APMSF	58	153
Factor Xa	27	44
Factor Xa + p-APMSF	84	291

Individual preparations were pretreated with a 600-mol excess of p-APMSF at 25°C for one hour and dialyzed overnight against 3 one-liter changes of 20 mM Mes-Tris, pH 6.0. 0.1 ml samples of factor X (1 umol/ml) or factor Xa (11 nmol/ml) were assayed by APTT or NAPTT as described in the text using a factor VIII inhibitor plasma (45 Bethesda units) as substrate.

factor X-factor II, VII or IX combinations. The rationale for this approach to the FEIBA assay was the same as previously described. If there was any enhanced activity we wanted to know if the acitivity could be blocked by naturally occuring inhibitors during a 40 minute incubation in substrate plasma. In all experiments the proteins were run over Sephadex G-25 prior to testing in order to remove the benzamidine in the storage buffer. Prothrombin alone or in combination with factor X had no effect on the FEIBA assay at concentrations that were as high as 10 times the normal plasma concentration. In addition, factor VII had no ability to enhance or inhibit the APTT at plasma concentrations that could be expected after administration of PCC's. There was, however, some ability to reduce the APTT at concentrations of factor VII that were 20 to 30

times normal. Factor IX alone, on the other hand, was able to signifigantly correct the APTT at 1 minute and in combination with factor X was able to enhance the factor X activity (Table 9). However, after 40 minutes of incubation, the factor IX acitivity was lost and the only apparent activity in the factor IX-X combination was that due to factor X. This is based on a comparison of the factor IX-X clotting time with that of factor X alone. These results suggest that the apparent activity was due to contamination with factor IXa and not to enhanced reactivity of factor IX with factor X.

Table 9. Effects of II, IX and VII on the FEIBA Assay

	APTT (sec)	
	1 min	40 min
Blank	96.8	
Factor X	51	60
Factor IX	61.6	86
Factor VII	87.8	97.2
Factor II	95	
Factor X + IX	37	51
Factor X + VII	53	63
Factor X + II	49	

The FEIBA assay was performed according to the procedures outlined in the methods section. The following concentrations of each of the vitamin K dependent factors was used: factor X (0.4 U/ml), factor IX (0.4 U/ml), factor VII (0.6 U/ml) and factor II (0.67 umol). In the mixing experiments all factors were kept constant at the concentrations indicated.

Prothrombinase Assay

It has been well established that certain criteria exist for the normal physiologic activation of the prothrombin molecule. These include Ca^{+2}, V(Va), phospholipid and Xa. We postulated that if factor X zymogen had intrinsic activity in the FEIBA assay then this activity would most likely be mediated via the components of the prothrombinase complex. In addition, if there was a difference in the kinetic constants of the X(Xa) mediated reactions then this would indicate a difference in the enzyme characteristics between factor X and Xa. We were specfically interested in showing the following characteristics; 1) that the K_m of factor X for prothrombin was different than the K_m of factor Xa for prothrombin. 2) When lipid was the limiting factor, factor X could compete with Xa for lipid binding sites. This would be reflected as a decrease in the rate of p-nitroanaline release, apparent inhibition. 3) The rate of release of p-nitroaniline, assumed to reflect activation of prothrombin, would not be affected when factor X was incubated with p-APMSF or AT-III but would be drastically decreased in the case of factor Xa.

Kinetics of the Prothrombinase Complex

In order to determine the K_m and V_{max} for the factor X and factor Xa system the lipid, Ca^{+2} and Va concentrations were constant at 15 uM of lipid, 2.5 mM $CaCl_2$ and 1 U/ml of factor Va respectively. In all cases the concentration of

prothrombin was varied between values of 1/10 the predicted K_m and 10 times the K_m. The concentration of Xa was kept constant at 2-5 nM and the concentration of factor X was 0.5 uM. Factor Xa gave values of 0.28± 0.19 uM for the K_m and 12.6 ± 4.3 umol thrombin/min for the V_{max}.

Several difficulties arose in determining the kenitic constants for factor X. The initial values obtained, K_m 0.21 ± 0.12 uM and V_{max} 11.6 ± 3.5 nM thrombin/min, although demonstrating a 1000 fold difference in the V_{max}, were not significantly different when the K_m values were compared with that for Xa (Figure 7). This could be due to contamination with Xa. To eliminate this possibility factor X was incubated with p-APMSF as previously described in this section. The control consisted of an aliquot of factor X incubated with methanol and an aliquot of factor Xa treated with both p-APMSF and methanol. After the samples had been applied to a Sephadex G-25 to remove excess inhibitor the proteins were immediately assayed for prothrombinase activity in order to determine kinetic constants. This particular procedure was followed to prevent autocatalytic activation of factor X which appeared to be an important variable if prolonged dialysis was used to remove the inhibitor. The results of this experiment gave a K_m for factor X cleavage of prothrombin of 2.6×10^{-8} M which was different by one order of magnitude from the K_m for Xa cleavage of prothrombin (Figure 8). Incubation of the Xa control with p-APMSF again resulted in almost complete lose of activity and the factor X-methanol control demonstrated a

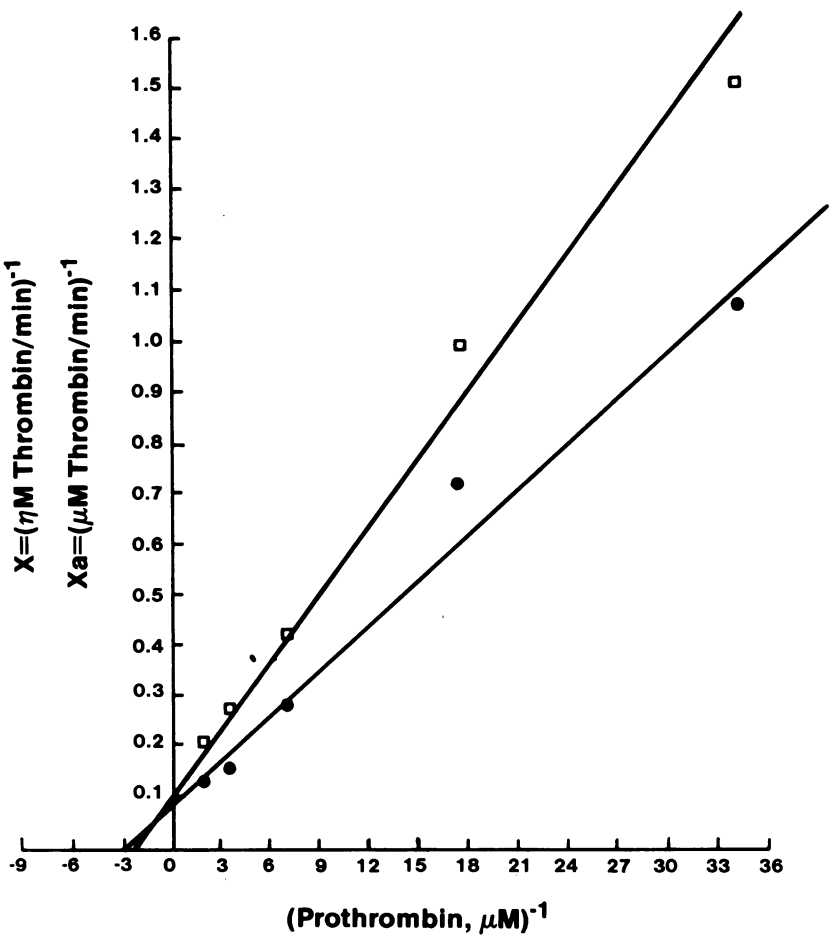


Figure 7. Double reciprocal plot of the initial velocity as a function of the prothrombin concentration. The assay mixture in a total volume of 1 ml consisted of factor Xa, 5.5 nmol/ml (●) or factor X, 0.5 umol/ml (□), prothrombin 0.03 to 0.59 umol/ml and 15 umol/ml of phospholipid. Calcium chloride, factor Va and S-2238 concentrations were as described under "Methods." The results shown are averages of 5 determinations for factor Xa and 3 determinations for factor X.

K_m value very similiar to the results obtained for factor X that had not been preincubated with p-APMSF.

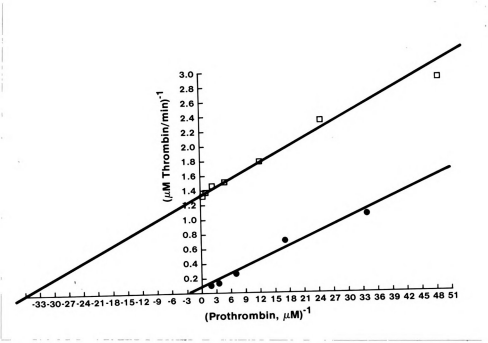


Figure 8. Double reciprocal plot of the initial velocity as as a function of the prothrombin concetration. The assay conditions were identical to those described in figure 7 with the exception of the factor X concentration which was 0.35 umol/ml and the pretreatment of the factor X with p-APMSF followed by chromatography on Sephadex G-25. Factor X (□), Factor Xa (●).

Lipid Binding Study

In order to establish that factor X could in fact compete successfully with factor Xa for lipid binding sites we choose to conduct an expirement using limiting concentrations of phospholipid. In order to observe competitive binding between factor X and Xa rate limiting concentrations of phospholipid were necessary. A number of authors (141,142) have demonstrated the ability of native factor X to bind to the same kind of phospholipid surface as for prothrombin, factor Xa and factor Va. It appears

feasible, therefore, that factor X zymogen could compete with factor Xa for either substrate or accessory components and that under certain conditions where both protein species are present, the binding of zymogen to cofactors would be reflected in the pattern of steady state kinetics. Zur et al. have commented that in the combined presence of low activity zymogen and high activity enzyme and if an obligatory cofactor (i.e. phospholipid) is rate limiting, then the zymogen would act as an inhibitor (82). We carried out two sets of experiments. The first was conducted at low concentrations of phospholipid (0.05 umol/ml). As can be seen in figure 9, when Xa is held constant and factor X is varied there is a factor X dependent inhibition of the Xa rate reactions. The second experiment was conducted at a 10 fold higher concentration of phospholipid and the intent was to try and determine if the enzyme (Xa) and zymogen (X) activities were additive. Although there appeared to be a difference in the curve when compared to the low lipid concentration, it was not felt that the data conclusively demonstrated an additive effect. The primary reason for this lies in both the sensitivity of the assay system and the large difference in catalytic activity between factor X and Xa. In the chromogenic system the levels of factor X being used in this expirement do not show significant activity and are overshadowed by the Xa activity.

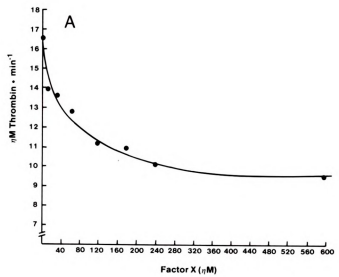


Figure 9. Inhibition of factor Xa enzymatic activity by factor X zymogen. The assay was performed in the presence of prothrombin (1.65 umol/ml), factor Xa (2.2 nmol/ml), factor X (0.0 to 0.59 umol/ml) and phospholipid (0.05 umol/ml). The other reactants were as described in the text.

Inhibition of Prothrombinase Activity With p-APMSF

Finally, we wanted to establish that the inhibition or lack of inhibition, of factor Xa and factor X, by p-APMSF and AT III was the same in the prothrombinase assay when compared to the FEIBA system. The initial experiments were conducted with a 600 mol excess of p-APMSF. However, because of the possibility of incomplete inhibition the inhibitor was increased to a final concentration of 2 mM. Both inhibitor concentrations gave similiar results. We observed inhibition of the initial reaction rate of factor Xa-prothrombinase activity, expressed as change in optical density in the chromogenic assay, equivalent to approximately 95% inhibition (Figure 10A). The same excess of

p-APMSF evidenced virtually no inhibition of the initial reaction rate of factor X zymogen (Figure 10B). It is to be noted that following pretreatment of the native zymogen with p-APMSF, there was no significant lose of procoagulant activity when the zymogen was assayed in an RVV-phospholipid activating system. This indicated that this particular inhibitor, at the concentrations being used, did not bind to the serine active site of the zymogen.

Inhibition of Prothrombinase Activity with AT III

Since factor Xa reacts with AT III with loss of activity, we compared the reaction of active enzyme and zymogen with the inhibitor. We employed experimental conditions whereby the inhibitor is removed from the assay system to avoid inhibition of the thrombin product. This was accomplished by incubating factor Xa or zymogen with AT III-Sepharose following which the residual prothrombinase activity was determined. It is seen from Figure 11 that both activated factor X and zymogen lost enzymatic activity in the presence of AT III-Sepharose although the degree of activity lost by the zymogen was considerably less than the active enzyme. Quantitatively, the initial rates of reaction, expressed as change in optical density in the chromogenic assay, were inhibited 99% and 65% for factor Xa and zymogen, respectively. Several variables could affect the outcome of the experiment. 1) Contamination of the system with AT III leaching off the Sehparose. This

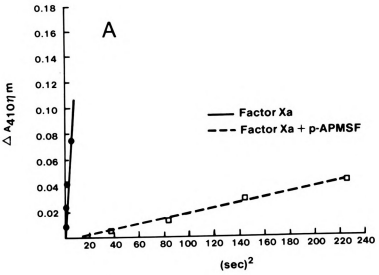
particular problem would be reflected most significantly in the factor X rate reaction because of the greatly decreased rate of prothrombin activation and the prolonged interval of time during which AT III could inactivate the IIa product. 2) Non-specifc adsorption of factor X to AT III-Sepharose would result in an apparent loss in activity. Although the major issue in this experiment was to demonstrate a difference in the inhibition of factor X and Xa, nonspecfic adsorption might be better demonstrated by first inactivating AT III with 1,2-cyclohexanedione and then attaching it to the Sepharose-4B. This control might help distinguish any effect not due to specfic binding of AT III to the active serine site.

Two additional observations have been made that are based on differences in the factor X-Xa reaction. First, when assaying, in a FEIBA system, for the Xa or X mediated correction of the APTT a log-log plot of correction in seconds versus Xa activity results in a linear relationship. In contrast to this the same plot of factor X results in a curvilinear relationship with maximum activity at approximately 55 seconds. The activity appears to plateau at this level and increasing factor X concentrations do not result in any significant improvement in the APTT. It is possible that this difference is due to a much slower cofactor mediated activation of prothrombin. Specifically, IIa generation is considerably slower and the dependent activation of V to Va could very likely reflect this rate difference. The fact that Va generation plays a significant

role is demonstrated by the observation that trace amounts of IIa (concentrations not high enough to alter the FEIB activity by themselves) will potentiate the factor X mediated improvement in the APTT (86).

The second observation is based on examining the time course of development of prothrombin activation products during both a factor X and factor Xa kinetic analysis. In order to monitor both the prothrombin cleavage products and the release of p-nitroaniline from the chromogenic substrate two identical reactions were run. The release of p-nitroaniline was monitored as described in the methods section under "Prothrombin Activation Assay". The prothrombin activation fragments were monitored by stopping the reaction at timed intervals by mixing 0.2 ml of the reaction mixture with 0.2 ml of 0.01 M phosphate buffer pH 7.2 containing 20 mM EDTA and 1% SDS. Each sample was then placed in a boiling water bath for one minute and 100 ul of this mixture was applied to 7.5% SDS-PAGE gels.

Activation of prothrombin by factor Xa, in combination with lipid, Ca^{+2} and factor Va, resulted in the development of prothrombin intermediate 2, thrombin and a small amount of intermediate 1 within two minutes of the initiation of the reaction. The production of intermediate 2 as the major cleavage product is consistant with the presently accepted mechanism for cleavage of prothrombin by factor Xa. Small amounts of intermediate 1 would be expected in an in vitro system as a result of autocatalytic activation of prothrombin by thrombin.



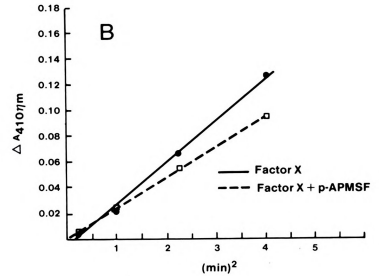


Figure 10. The effect of the inhibitor p-APMSF on the time course of prothrombin activation by factor Xa (A) or factor X zymogen (B). Individual preparations were pretreated with a 600-mol excess of p-APMSF at 25°C for one hour and dialyzed overnight against 3 one-liter changes of 20 mM Mes-Tris, pH 6.0. The assay was performed in the presence of factor Xa (5.5 nmol/ml or factor X (0.7 umol/ml) and prothrombin (0.67 umol/ml). All other conditions are as given in the legend to figure 7.

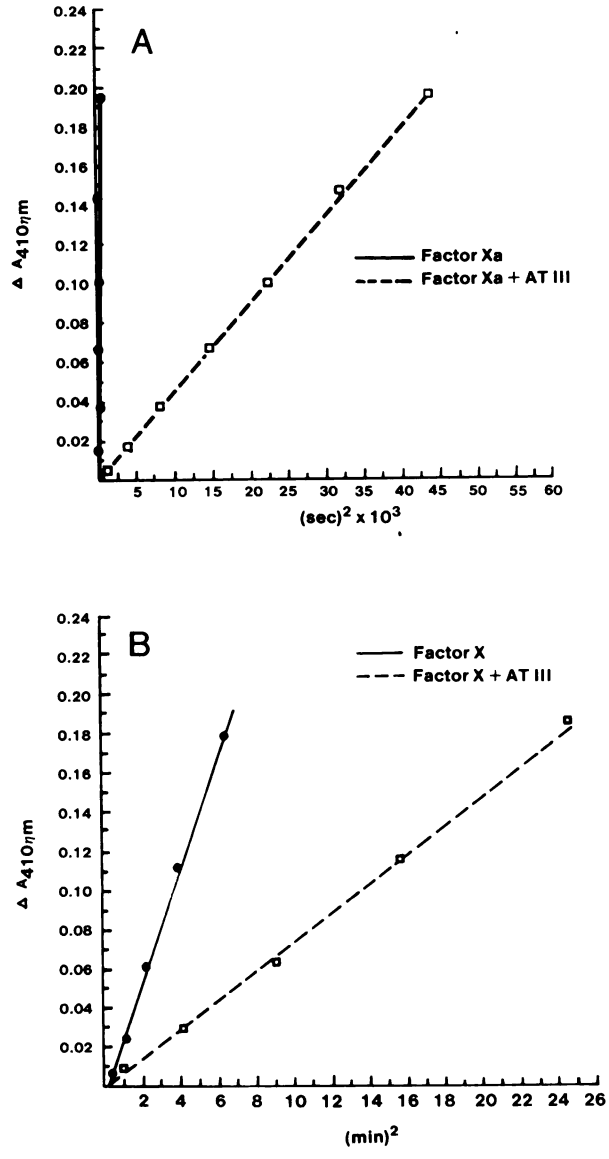


Figure 11. The effect of AT III-Sepharose on the time course of prothrombin activation by factor Xa (A) of factor X zymogen (B). Experiments were performed in the presence of equal volumes of AT III Sepharose (9 mg protein/ml) and either factor Xa or factor X zymogen. The reactants were incubated at 25°C for 60 min and the AT III-Sepharose removed by filtration on a mini-column (1.4 x 6.5 cm). The final reaction mixture contained factor Xa (8.6 nmol/ml), or factor X zymogen (0.27 umol/ml), prothrombin (0.16 umol/ml) and phospholipid (15.0 umol/ml), All other conditions are as described in the text. A control reaction mixture consisted of Sepharose that had been activated and treated in a manner identical to the AT III-Sepharose that had been activated and treated in a manner identical to the AT III-Sepharose but without the addition of the ligand.

When factor X was substituted for Xa in the prothrombinase complex the cleavage products that developed were markedly different in both the time course of development and the significance of the major band. In this case, after 10 minutes of incubation of the reaction mixture the only apparent change in the prothrombin molecule was the presence of a very faint band corresponding to intermediate 1. After 20 minutes of incubation there was still very little change in the prothrombin band but traces of intermediate 2 and IIa were present. The trace of intermediate 1 seen after 10 minutes of incubation of prothrombin with factor X is most likely due to contamination of the system with IIa. Although IIa is removed by adsorption onto benzamidine-Sepharose following activation of factor V it is likely that trace amounts are still present and this could account for the presence of the intermediate 1 band. The lack of an identifiable IIa band after prolonged incubation with factor X could be related to the lack of sensitivity of the PAGE technique when trying to detect small amounts of proteolytic activation products. Alternatively, a stoichiometric alteration in the prothrombin molecule could occur as a result of binding to the proteins of the prothrombinase complex resulting in an enhanced ability to cleave the chromogenic substrate and/or fibrinogen. The second possibility is similiar to the mechanism of the reported activation of prothrombin by staphylocoagulase or streptokinases activation of plasminogen (160-162). It would seem more likely, however, that the decreased rate of

reaction is due to the slow generation of small amounts of IIa.

DISCUSSION

This work was initiated in an attempt to find a plausable explanation for the ability of the prothrombin complex concentrates to cause hemostasis in hemophiliacs with an inhibitor to factor VIII. The specific approach to the problem was based on the observation of Tishkoff that factor X and/or factor X in conjunction with IIa would correct the APTT or NAPTT of factor VIII deficient plasma that contained an inhibitor to factor VIII (86). The data presented in this paper supports the contention that factor X zymogen is the mediator of the coagulant activity observed in an intrinsic clotting assay system and can bypass the factor VIII inhibitor in vitro. It was speculated that the coagulant activity of native factor X was probably not attributable to the presence of trace contamination with active enzyme since preincubation of the zymogen with factor VIII inhibitor plasma, which contains a normal amount of AT III activity, resulted in incomplete inhibition of coagulant activity. The present study expands on this observation to include in-depth studies of the direct effect of AT III and a synthetic, highly specific, protease inhibitor, p-APMSF, on both the coagulant activity and steady state kinetics of factor X. In addition, the factor X molecule is critically examined for any possible alterations or cross contamination

with Xa. The effect of additional coagulant factors, II, VII and IX are evaluated, the V_{max} and K_m for factor X is determined and the activation fragments of II are evaluated for both a factor X and Xa catalyzed reaction.

Enzymatic activity has been reported previously for other blood clotting zymogens such as factor VII and factor XII, although some investigators have questioned a physiologic role for these relatively low-activity zymogens (163-164). Noteworthy in this regard are the observations of Kerr et al. on the existence of a preformed catalytic apparatus in the factor X zymogen as determined by the incorporation of specfic organic flouride inhibitors (165). In addititon, recent work by Fair and Bahnak has demonstrated the existance of a single chain form of factor X that is produced by human hepatoma cells and then cleaved to the two chain form that is most often identified in plasma (166). In light of this observation, it may be necessary to reevaluate what is presently thought of as the mechanism of factor X activation. It would appear that not only is the cleavage of a small molecular weight peptide from the amino terminal end of the heavy chain required for factor X activation but, in addition, the factor X is first cleaved to a heavy and light chain structure. Therefore, factor X may exist in native plasma as what could be thought of as an intermediate form between the native, single chain, zymogen and the active enzyme form, Xa. This finding coupled with the fact that factor X is the only protein of the prothrombin complex that exists as a heavy and light

chain structure might suggest a rather unique function for this intermediate form of the factor X molecule.

If the intermediate form of the factor X molecule is capable of catalytic activity it would be important to understand what conditions must exist for this activity to be expressed and what physiologic significance this activity has. Several different possibilities were considered to explain the in vitro proteolytic activity of the factor X zymogen. 1) Factor X zymogen is contaminated with factor Xa; 2) The zymogen enzymatic activity is attributable to an, as yet, unidentified intermediate of factor X present in low concentration; 3) Factor Xa catalyzes a feedback reaction on the substrate factor X, particularly in the presence of phospholipid and calcium; 4) Factor X zymogen has intrinsic enzymatic activity.

To evaluate the first mechanism we used specfic, active site directed, inhibitors known to affect factor Xa activity. As can be seen from Figure 10 and Table 8 p-APMSF had no significant effect on either enzymatic rate reactions or on the coagulation assays. It should be noted that special precautions were taken to eliminate possible cross contamination of the zymogen preparations with active enzyme and also to prevent autocatalytic activation of factor X. We also controlled for the short half-life of p-APMSF as well as the effect of protein concentration on the rate of inhibition. We did not observe any change in the factor X activity when determined by the RVV activating assay.

Although it has been shown that a decrease in the factor X activity occurs after incubation with some fluoride inhibitors (165) it was felt that because of the variation in the molecular structure of the inhibitor used in these experiments (p-APMSF) no significant comparisons could be drawn between our results and those using methanesulfonyl fluoride as the inhibitor.

The AT III data shown in Figure 11 suggests one of two possibilities. Either the apparent slow rate of inhibition of the zymogen as compared with the active enzyme is due to a different kind of zymogen-antithrombin III interaction or, non-specifc adsorption of factor X to the Sepharose-AT III had occured. The latter possibility does not seem as likely as protein concentration before and after adsorption with AT III were almost identical. Antithrombin III was used in our study at much higher concentrations than those that are known to inhibit serine proteases of the coagulation system. Elsinger (78) has presented data that heparin plus AT III does not inhibit FEIBA under conditions where factor Xa is totally inhibited, and similiar findings have been reported by Barrowcliffe et al. (80).

As another possible explanation for the apparent prothrombinase activity of factor X zymogen we attempted to identify trace component derivaties of the factor X zymogen that might account for the enzymatic activity. Recognizing that the limitations of analytic assay by SDS-PAGE are such that we were unable to visualize protein contaminants present in less than a 1% concentration, we found no

alteration in the factor X molecule on either normal gels or gels that had been loaded with excessive amounts of protein. In addition, the factor X was analyzed by amino-terminal sequencing to determine if any alteration in the protein had occurred as a result of our protein isolation technique. The results were consistant with the published literature and did not indicate any amino terminal alteration of factor X (148). Beta factor X would be a suitable candidate for an intermediate, enzymatically active, form of factor X and is formed by the cleavage of a small glycopeptide from the carboxyl-terminal end of the heavy chain of factor X by factor Xa. Although we have not sucessfully identified a zymogen intermediate, no zymogen of the coagulation system is known to be activated by COOH-terminal cleavage.

A factor Xa feedback mechanism, occurring during the course of the reaction, could play a role in either the coagulation system or the steady state kinetics. Van Dieijen et al. (167) studied the factor Xa feedback mechanism of purified bovine proteins under optimal conditions. They found no extra factor Xa generated when Xa was incubated with factor X, calcium ions and phospholipid. Likewise, factor Xa and factor X were not seen on polyacrylamide gels in the presence of sodium dodecyl sulfate after 30 minutes of incubation. Noteworthy in this regard, Morrison and Jesty (168) did not observe release of activated peptide by brief treatment of tritiated factor X by Xa and moreover, Mertens and Bertina have shown that the action of factor Xa

on human factor X generates an enzymatically inactive derivative (169).

Initial experiments that were conducted to differentiate the kinetic activity of factor X and Xa showed a 1000 fold variation in the maximum velocity but only a two to three fold difference in the K_m. We had expected that if the factor X molecule was different in its enzymatic activity from Xa there should be a significant difference in the K_m for the enzyme and zymogen. It was hoped that this difference would be at least one order of magnitude. The fact that the K_m's were so close suggested the possibility of trace contamination with Xa or autocatalytic byproducts of factor X that could develop during the preincubation of the zymogen with p-APMSF and the subsequent dialysis to remove the inhibitor. In order to test this the mixture of inhibitor and factor X or Xa were applied to a Sepharose G-25 column immediately following the preincubation to remove the inhibitor. It was felt that by eliminating the lengthy dialysis prior to testing, the possibility of autocalalytic acitvation or cleavage of factor X could by minimized. The Lineweaver-Burke analysis of data from this experiment gave a K_m that was approximately one order of magnitude different than the enzyme Xa. It should be noted, however, that the K_m varied in a direction that was opposite to what would have been predicted. Rather than the zymogen form requiring more substrate to reach half-maximum velocity it required less. This variation could be accounted for based on differences in the stoichiometry of the proteins involved in

the prothrombinase complex. Because factor X has, at this point, not undergone amino-terminal cleavage to the factor Xa molecule steric interference could limit the amount of prothrombin capable of binding to the complex. This in turn would be reflected in an apparent decrease in the K_m for the prothrombin substrate.

The expression of zymogen prothrombinase activity may have physiologic implications from our observation that the zymogen competes with factor Xa for phospholipid binding sites (Figure 9). This data is also consistent with our observation that phospholipid is obligatory for apparent zymogen enzymatic activity and, in addition, explains why increasing concentrations of factor X will inhibit the factor Xa clotting activity (data not shown). As can be seen in figure 10 as the concentration of factor X was increased there was a decrease in the rate of the enzymatic reaction with factor Xa that reached a maximum at approximately 0.36 umol of factor X. Further increases in factor X did not result in a decrease in the reaction rate.

We have observed a total absence of factor X peptidase activity toward the factor Xa chromogenic substrate S-2222 within the sensitivity of our experiments (data not shown). Notwithstanding, we propose that apparent zymogen enzymatic activity demonstrated under experimental conditions in this paper reflects the enhanced reaction rate of the prothrombinase complex that may result from concentration on the phospholipid vesicle surface of the enzyme (factor X)

and substrate (prothrombin). We conclude from the present studies that the apparent enzymatic activity of the zymogen can initiate clotting under conditions where plasma levels of factor X are markedly elevated as occurs postinfusion in patients treated with PCC.

APPENDIX

APPENDIX 1

The kinetic evaluation of the activation of prothrombin was based on an assay system developed by Kosow et al. (160,170). This assay is a continuous spectrophotometric assay and monitors the release of p-nitroaniline from the thrombin specific substrate S-2238. The Lineweaver-Burke plots that were obtained from this assay system were linear, indicative of Michaelis-Menten kinetics.

The molar extinction coefficient (ε) of p-nitroanilide is 9400 $M^{-1}cm^{-1}$ at 410 nm. This is based on the fact that a 1M solution of p-nitroaniline in a cuvette with a 1 cm light path will give an absorbance at 410 nm of 9400. Therefore, the absorbance of a solution of p-nitroaniline at 410 nm divided by 9400 gives the molarity of p-nitroaniline.

The first-order rate constant (k) for the hydrolysis of S-2238 by IIa is the moles of p-nitroaniline released per mole of thrombin per unit time. This reaction is a simple single enzyme substrate reaction of the form:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E$$

where E is enzyme, S is substrate, ES is an intermediate enzyme substrate complex and P relates to the production of product. k_1, k_{-1} and k_2 are rate constants and describe how the concentration of the enzyme-substrate will change with time. Therefore, the rate at which ES will be formed is described by $k_1[E][S]$, the rate of formation of product is described as $k_2[ES]$ and finally, the rate of conversion of ES back to E and S would equal $k_{-1}[ES]$. An equation can be written that will define the change in [ES] as a result of the change in time.

$$d[ES]/dt = k_1[E][S] - k_{-1}[ES] - k_2[ES] \qquad (1)$$

In order to derive the Michaelis-Menten equation a term that describes total enzyme concentration $[E_t]$ is required. This is expressed as:

$$[E_t] = [E] + [ES] \qquad (2)$$

which when used to express [E] can be be rearranged to:

$$E = [E_t] - [ES] \qquad (3)$$

If this value is substituted into equation (1) the rate of change in [ES] expressed as a function of t becomes:

$$d[ES]/dt = k_1([E_t] - [ES])[S] - k_{-1}[ES] - k_2[ES] \quad (4)$$

or

$$d[ES]/dt = k_1([E_t] - [ES])[S] - (k_{-1} + k_2)[ES] \quad (5)$$

If the steady state assumption of Briggs and Haldane (171) is used then the rate of change of [E] and [ES] with time is zero in comparison to the rate of change of either [S] or [P] and therefore:

$$d[ES]/dt = 0 \quad (6)$$

and,

$$k_1([E_t] - [ES])[S] - (k_{-1} + k_2)[ES] = 0 \quad (7)$$

expanding this eaquation gives:

$$k_1[E_t][S] - k_1[ES][S] - k_{-1}[ES] - k_2[ES] = 0 \quad (8)$$

$$k_1[E_t][S] = (k_1[S] + k_{-1} + k_2)[ES] \quad (9)$$

and solving for [ES] results in:

$$[ES] = \frac{k_1[E_t][S]}{k_1[S] + k_{-1} + k_2} \quad (10)$$

dividing by k_1 leaves

$$[ES] = \frac{E_t[S]}{[S] + k_{-1} + k_2/k_1} \quad (11)$$

From the first rate constant discussed it is known that $v = k_2[ES]$ therefore, if you multiple through by $k_2$the expression becomes:

$$v = \frac{k_2[E_t][S]}{[S] + k_{-1} + k_2/k_1} \quad (12)$$

The expression in the denominator that contains only rate constants is referred to as the K_m and in the special instance in which the [S] is equal to the K_m the reaction velocity is at half maximum. If the substrate concentrations were very large relative to the K_m, then:

$$v = k_2[E_t] \tag{13}$$

and at very high substrate concentrations the maximal velocity V_{max} is approximated. Therefore:

$$V_{max} = k_2[E_t] \tag{14}$$

if this expression is substituted into equation (12) it becomes:

$$v = \frac{V_{max}[S]}{K_m + [S]} \tag{15}$$

This is the Michaelis-Menten equation and describes a rectangular hyperbola. Although this equation allows for the determination of the V_{max} and K_m for a particular enzyme substrate reaction it is more difficult to use for analysis. Linear transformations have been developed and the most common of these in the Lineweaver-Burke or double-reciprocal plot.

$$1/v = 1/V_{max} + K_m/V_{max} \quad 1/[S] \tag{16}$$

Where 1/v is plotted vs. 1/[S] and the y intercept is equal to $1/V_{max}$ and the x intercept is $-1/K_m$. This particular plot was choosen as it is most often used in enzymology

although it may not be as statistically valid as other linear transformations.

Based on these equations, the first order rate constant for IIa cleavage was determined using a constant enzyme concentration of 5.8 nmol/ml of IIa and a substrate concentration that varied form 1.6×10^{-4} mol/ml to 2.1×10^{-5} mol/ml. The V_{max} obtained from a double reciprocal plot of this data was then fitted into the equation:

$$k = \frac{(A_{410}/\text{sec})/9400}{\text{mmoles IIa}} \qquad (17)$$

The reaction mixture in this case was in a total volume of 1 ml and duplicates the buffer system used in the prothrombinase assay.

Factor IIa catalyzes the cleavage of H-D-Phe-Pip-Arg-pNA (S-2238) and this cleavage is monitored at 410 nm. The production of IIa is catalyzed by factor Xa, a serine protease, and this activation is markedly accelerated by the addition of factor Va, phospholipid, and Ca^{+2} to from what is referred to as the prothrombinase complex. When S-2238 is added to the reaction mixture their is a parabolic increase in absorbancy with time that is indicative of a constant rate of IIa production. This curve conforms to the equation:

$$x = v_o t + 1/2at^2 \qquad (18)$$

where x is absorbancy, v_o is the velocity at time zero and in this system correlates with endogenous activity or activity not due to specific enzyme addition, t is time and

a is acceleration. This equation is used to describe motion with constant acceleration. When t = 0 the instantaneous speed v is v_o and since acceleration is assumed to be constant v will increase in proportion to the time. If we were considering a straight-line speed-time graph the average acceleration would equal $\Delta v/\Delta t$. If this was applied to an entire time internal t_o to t, which would correspond to v_o and v respectively, the average acceleration would be replaced with a constant value for acceleration where:

$$a = \frac{v - v_o}{t} \tag{19}$$

or

$$v = v_o + at \tag{20}$$

This equation, although describing a straight speed-time graph, does not describe the parabolic curves obtained during the activation of prothrombin. In this particular instance because the rate of increase of speed is uniform the average speed over an interval of this curve is $(v_o + v)/2$. The change in absorbancy (x) or the distance covered is expressed then as the average speed multiplied by the elapsed time.

$$x = \frac{v_o + v}{2} t \tag{21}$$

If equation 20 is substituted for v:

$$x = \frac{v_o + (v_o + at)}{2} t \tag{22}$$

or

$$x = v_o t + 1/2at^2 \qquad (23)$$

This equation conforms to the equation for a straight line, $y = a + bx$, and allows the determination of acceleration or change in absorbancy in a given time. If you subtract the endogenous activity of the mixture ($v_o t$) from the absorbancy (x) and plot this value vs. t^2 you obtain a straight line with a slope (b) equal to a/2. This equation is valid only as long as IIa is being produced at a constant rate. Therefore, the reactions were followed for only short periods of time and only data having a correlation coefficient of greater then 0.995, as determined from the plots of abosrbancy vs. t^2, was used. The slope of the line obtained from this plot, which equals a/2, is proportional to the rate of IIa formation.

This value was then used to determine the velocity of the reaction via the equation:

$$(M)/t = a/(\varepsilon k/2) \qquad (24)$$

where a is acceleration, ε is the extinction coefficient of p-nitroaniline and k is the first order rate constant for the cleavage of S-2238 by IIa.

The concentrations of phospholipid, Va, Ca^{+2} and enzyme or zymogen were kept constant as described in the "Materials and Methods" section and the concentration of II was varied. The velocity of the reaction was determined at each concentration of II and the K_m and V_{max} for either enzyme or

zymogen was obtained from a double reciprocal plot of the velocities and substrate concentrations.

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