Formation of a Serine Enzyme in the Presence of Bovine Factor VIII (Antihemophilic Factor) and Thrombin

Abstract. *Factor VIII is present in plasma in a precursor or inactive form. When bovine factor VIII that has been purified approximately 10,000-fold is incubated with thrombin, an activated product is formed which participates in the conversion of factor X to factor X_a in the presence of factor IX_a, calcium ions, and phospholipid. This activated product, which has been tentatively identified as activated factor VIII, was stable when formed in the presence of 0.25*M *$CaCl_2$ but was rapidly inactivated in the absence of $CaCl_2$. It was inhibited by diisopropyl phosphorofluoridate and antithrombin III, suggesting that it is a serine enzyme. The exact role of this serine enzyme in the intrinsic pathway of coagulation remains to be established.*

Factor VIII (antihemophilic factor) is a plasma protein that is inactive or absent in individuals with classic hemophilia (*1*). It participates in the middle phase of blood coagulation where it is involved in the activation of factor X (Stuart factor) (*2*). This reaction, which occurs in the intrinsic pathway of blood coagulation, can be illustrated as follows:

$$\text{factor X} \xrightarrow[\text{phospholipid}]{\text{factor VIII}\ \text{factor IX}_a,\ Ca^{2+}} \text{factor X}_a$$

Lundblad and Davie (*3*) and Macfarlane *et al.* (*4*) suggested that factor VIII participates as an enzyme in this reaction. Other evidence, however, has been consistent with the idea that factor VIII forms a complex with factor IX_a (activated Christmas factor) in the presence of calcium ions and phospholipid (*5*) and that this complex converts factor X to factor X_a (*6*). In the activation of factor X, a peptide bond between arginine-51 and isoleucine-52 is cleaved in the heavy chain giving rise to factor X_a (*7*). Since factor IX_a is a serine esterase (*8*), it has been suggested that factor IX_a is the enzyme in this reaction while factor VIII is a regulatory protein (*2*). Minor proteolysis of highly purified preparations of factor VIII by enzymes such as thrombin greatly increases its coagulant activity (*9*). The mechanism for this reaction, however, has not been established.

We now report that a serine enzyme is formed when bovine factor VIII is incubated with thrombin, and that this enzyme is inactivated by inhibitors such as diisopropyl phosphorofluoridate or antithrombin III.

The activation of bovine factor VIII by thrombin in the presence and absence of 0.25*M* $CaCl_2$ is shown in Fig. 1. This concentration of $CaCl_2$ was first used by Owen and Wagner (*10*) in their experiments dealing with the dissociation of canine factor VIII. In the absence of calcium, a rapid increase in coagulant activity was observed followed by a substantial rate of inactivation. After 1 hour, a small residual activity remained, and this activity was stable and similar in amount to that of the factor VIII activity initially added. The overall activation of factor VIII in the absence of calcium was about 100-fold when the amount of thrombin used was 0.3 μg per milliliter of reaction mixture. This concentration of thrombin is higher than that required for maximum activation of factor VIII, but was used so that we could compare these experiments with those carried out in the presence of $CaCl_2$. Factor VIII was activated about 25-fold with thrombin (0.3 μg/ml) in the presence of 0.25*M* $CaCl_2$. This activity was very stable and was the same after 3 hours of incubation (Fig. 1). Thus, the presence of calcium prevented maximum activation of factor VIII, but the activated factor VIII was stable once it was formed.

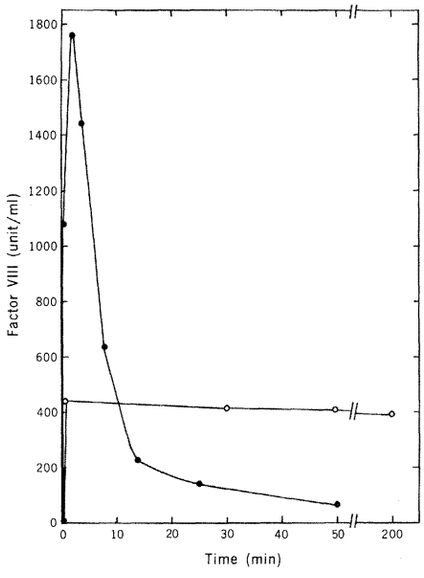

Fig. 1. Activation of bovine factor VIII by thrombin in the presence and absence of $CaCl_2$. The incubation mixture contained factor VIII (0.7 mg/ml) and thrombin (0.3 μg/ml) in 0.05*M* tris-HCl buffer, *p*H 7.35, containing 0.01*M* NaCl or 0.25*M* $CaCl_2$ and 0.01*M* NaCl. Portions were removed at various times, diluted 100- to 2000-fold in 0.05*M* tris-HCl buffer, *p*H 8.0, and immediately assayed for coagulant activity as described (*20*). Factor VIII was prepared by the method of Legaz and Davie (*20*) and migrated as a single band on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. Bovine thrombin was prepared by the method of Thompson and Davie (*21*) and also showed a single, sharp band on SDS polyacrylamide gel electrophoresis. Closed circles, no $CaCl_2$; open circles, $CaCl_2$ present.

Factor VIII was then examined before and after activation for its sensitivity to serine protease inhibitors. Factor VIII was not affected by incubation for 3 hours with high concentrations of diisopropyl phosphorofluoridate (0.01*M*) or antithrombin III. It was readily inhibited, however, after activation by thrombin (Fig. 2). In these experiments, factor VIII was activated about 25-fold with thrombin, and then portions of the mixture were incubated with 0.005*M* diisopropyl phosphorofluoridate or antithrombin III. In each case, a rapid inhibition of the activated clotting factor (tentatively called activated factor VIII) occurred. Furthermore, the inhibition of activated factor VIII by antithrombin III was accelerated by the addition of a very low concentration of heparin. Heparin alone at such a concentration (0.0004 unit/ml) had no effect on activated factor VIII, but higher concentrations of heparin (0.002 unit/ml) readily inhibited activated factor VIII in the absence of antithrombin III. The total inhibition of activated factor VIII was about 85 percent in the presence of diisopropyl phosphorofluoridate or antithrombin III. Some residual coagulant activity always remained, however. This coagulant activity may not be factor VIII since the addition of more thrombin to a dialyzed sample previously inhibited by diisopropyl phosphorofluoridate resulted in only a small additional activation of the preparation.

Factor VIII activated by thrombin in the presence or absence of 0.25*M* $CaCl_2$ readily corrected factor VIII–deficient plasma and was also required for the activation of factor X in the presence of factor IX_a, calcium ions, and phospholipid, as previously reported (*6*). Activated factor VIII had very little or no effect, however, on factor IX–deficient plasma or factor X–deficient plasma. Furthermore, it did not clot fibrinogen when examined in concentrations 100 times higher than the concentration used in the coagulation assay with factor VIII–deficient plasma.

A difference in the gel filtration pattern for factor VIII before and after thrombin activation was also noted (*11, 12*). The elution pattern for bovine factor VIII in the presence of 0.25*M* $CaCl_2$ is shown in Fig. 3A. Factor VIII activity appeared in

the void volume of the column with the protein peak. Previous experiments from our laboratory with bovine factor VIII have shown that this peak also contains platelet aggregating activity (von Willebrand factor) (*9*).

When factor VIII was activated for 5 minutes by thrombin, a major portion of the coagulant activity was retarded by the gel (Fig. 3B). After incubation for 3 hours, essentially all of the coagulant activity was retarded by the gel and eluted from the column in a position identical to bovine serum albumin (Fig. 3C). This suggests that the protein with coagulant activity has an apparent molecular weight of about 70,000. The recovery of coagulant activity after gel filtration ranged from 30 to 40 percent after concentration by ultrafiltration with an Amicon Diaflo concentrator with a PM-10 membrane. This fraction, which was quite labile, was also sensitive to diisopropyl phosphorofluoridate. The coagulant activity was eluted shortly before thrombin under these conditions and trailed slightly into the leading edge of the thrombin peak, as shown in separate experiments with ^{131}I-labeled thrombin.

The present experiments are consistent with the suggestion that factor VIII incubated in the presence of thrombin gives rise to a serine enzyme, and that this enzyme results from proteolysis of a precursor protein. The identity of this precursor protein is not known. The precursor molecule probably makes up only a very small portion of the factor VIII preparation used in these studies, however, since the amount of protein present in the activated factor VIII peak from the gel filtration experiments is extremely low. We assume that there is a reasonably effective conversion of the precursor protein to the serine enzyme and a limited amount of nonspecific cleavage and inactivation of the coagulant activity by thrombin. Thus, it appears likely that our bovine factor VIII preparations contain primarily von Willebrand factor in addition to one or more trace proteins with coagulant activity. Also, the platelet aggregating and coagulant activities in these preparations are probably owing to two distinct proteins that have been copurified. This conclusion is consistent with previous experiments showing the separation of factor VIII coagulant activity from von Willebrand factor by ion exchange chromatography, gel filtration, and transfusion experiments in vivo (*13*). Thus, the isolation of factor VIII in a precursor form may require a purification of a millionfold or more starting from plasma. Furthermore, the isolation and characterization of this precursor will be necessary to determine its true molecular weight. Elution of the coagulant activity in the void volume of agarose columns in the absence of 0.25*M* $CaCl_2$ suggests a molecular weight of greater than 10^6 for the precursor protein. This may be an artifact, however, due to extensive aggregation (*14*), binding of the coagulant activity to another high molecular weight protein (*15*), or nonspecific interaction of the protein with agarose (*16*). Rick and Hoyer (*17*) reported an *S* value of 6.7 for the human factor VIII that they isolated by gel filtration on agarose. This value is consistent with a molecular weight of about 150,000. This preparation, however, was retarded by gel filtration on agarose and may have undergone minor proteolysis. Indeed, the importance of proteolysis has been reported recently by Beck *et al.* (*18*) who used Trasylol in the isolation of factor VIII. The addition of this protease inhibitor prevented the appearance of low molecular weight factor VIII as measured by gel filtration on agarose. In similar experiments, however, Poon and Ratnoff (*19*) were unable to demonstrate an effect of protease inhibitors on the presence of low molecular weight factor VIII.

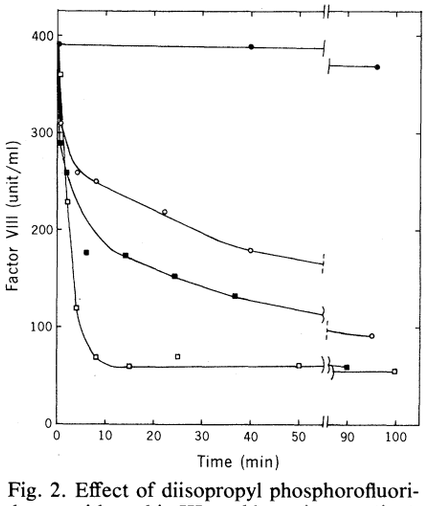

Fig. 2. Effect of diisopropyl phosphorofluoridate, antithrombin III, and heparin on activated factor VIII. Factor VIII (0.7 mg/ml) was activated with thrombin (0.3 μg/ml) in 0.05*M* tris-HCl buffer, *p*H 7.35, containing 0.25*M* $CaCl_2$ and 0.01*M* NaCl. Activated factor VIII was then incubated at 22°C with no additions (closed circles), 0.005*M* diisopropyl phosphorofluoridate (open squares), antithrombin III (22 μg/ml) (open circles), or antithrombin III (22 μg/ml) plus heparin (0.0004 unit/ml) (closed squares). Portions were removed at various times, diluted 100- to 2000-fold in 0.05*M* tris-HCl buffer, *p*H 8.0, and immediately assayed for coagulant activity. All mixtures were incubated at 22°C in 0.05*M* tris-HCl buffer, *p*H 7.35, containing 0.25*M* $CaCl_2$ and 0.01*M* NaCl. Antithrombin III was prepared from bovine plasma by heparin-agarose affinity chromatography (*22*).

Another possible explanation for the present results is that the serine enzyme is formed from an unknown coagulation factor distinct from factor VIII, and that this protein stimulates the coagulation process in the presence of factor VIII. Accordingly, the final identity of the serine enzyme as activated factor VIII can be established only after additional extensive purification.

The mechanism by which factor VIII is activated under physiological conditions is not known. Furthermore, little is known about how activated factor VIII may influence the activation of factor X. Experiments attempting to show by sodium dodecyl sulfate polyacrylamide gel electrophoresis an effect of activated factor VIII on factor X or factor IX_a in the presence or absence of calcium ions and phospholipid have been negative thus far. A trace amount of contaminating protein or proteins which may be a substrate for activated factor VIII and present in our factor X, factor IX_a, or factor

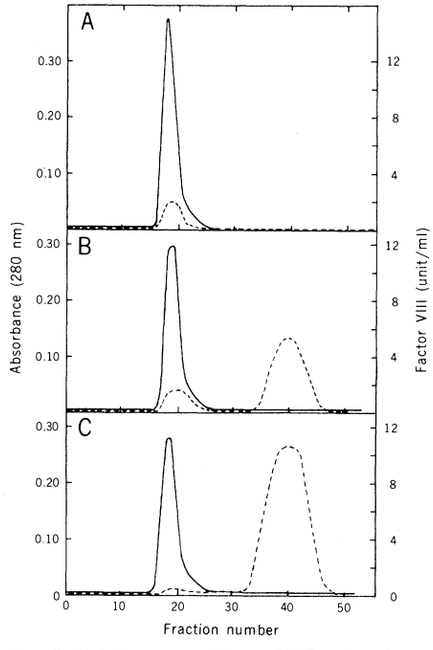

Fig. 3. Gel filtration of factor VIII and activated factor VIII on Sepharose CL-4B. A solution of factor VIII (1.0 ml containing 2.9 mg/ml) in 0.05*M* tris-HCl buffer, *p*H 7.35, containing 0.25*M* $CaCl_2$ and 0.01*M* NaCl was subjected to gel filtration on a Sepharose CL-4B column (1.5 by 30 cm) by a modification of the method of Cooper *et al.* (*23*). Protein was eluted with 0.05*M* tris-HCl buffer, *p*H 7.35, containing 0.25*M* $CaCl_2$, 0.01*M* NaCl, 0.005*M* benzamidine, and bovine serum albumin (0.5 mg/ml). Fractions (1.1 ml) were collected at 0°C at a rate of 0.5 ml per minute. The elution profile for factor VIII before activation is shown in (A). The elution profiles for activated factor VIII after 5 minutes and 180 minutes of incubation with thrombin are shown in (B) and (C), respectively. Solid lines refer to absorbance and dashed lines refer to coagulant activity.

VIII preparation cannot be ruled out.

The inhibition of thrombin-activated human factor VIII by diisopropyl phosphorofluoridate has been difficult to demonstrate. This is due in part to the rapid inactivation of the activated molecule even in the presence of high concentrations of $CaCl_2$. Similar results have also been observed by Switzer and McKee (*12*). Thus, the human preparation differs markedly from the bovine preparation in its stability after activation by thrombin.

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Antigenic Shift of Visna Virus in Persistently Infected Sheep

Abstract. *Visna viruses isolated from persistently infected sheep were antigenically distinct from the plaque-purified virus used for inoculation. The selection of antigenic variants under antibody pressure, thought to occur in vivo, was reproduced in sheep cell cultures inoculated with plaque-purified visna virus and maintained in antibody. Antigenic shift may be a mechanism for persistence of virus in slow or recurrent viral infections.*

Visna of sheep is one of the slow virus infections characterized by a prolonged incubation period and protracted disease (*1*). Unlike the etiologic agent of scrapie, the other prototype slow infection, the visna agent is a conventional virus that elicits an immune response in sheep and causes an inflammatory, demyelinating disease of the brain and spinal cord (*2*). Although visna virus is cytolytic for sheep cells in culture, the virus undergoes minimal replication in vivo (*3*).

The restriction of replication in sheep occurs immediately after inoculation, but virus can be isolated from many tissues—including peripheral blood leukocytes (PBL), by tissue explantation or cocultivation—for months to years after inoculation (*4, 5*). Furthermore, ease of virus recovery from PBL varies among individual animals but is not affected by the virus-neutralizing antibody which develops later in the infection (*6*). Regardless of the duration of the infection, tissues from infected sheep only occasionally yield cell-free virus, virus antigens are rarely found by immunofluorescence, and virus particles are not seen by electron microscopy (*4, 7*). These phenomena have been explained by the demonstration that visna virus, a retravirus containing an RNA-directed DNA polymerase, exists in vivo primarily as provirus (*8*). In contrast to some tumor-associated retraviruses, the proviral DNA does not hybridize with normal sheep DNA (*9*). In infected sheep, sequestration of the genome of visna virus as provirus may explain virus persistence in the immune animal; however, other mechanisms must be sought to explain the development of disease months to years after infection. The unpredictable development of disease might be explained by the intermittent release of antigenically altered virus which could escape neutralization in the immune host (*5*). To test this hypothesis, viruses isolated from sheep inoculated with visna virus were compared to the parent virus for possible antigenic shift.

Four Hampshire outbred lambs were inoculated intracerebrally with approximately 10^6 TCD_{50} (50 percent tissue culture infectious doses) of strain 1514 of visna virus which had been plaque-purified. Blood was taken from the sheep at regular intervals after inoculation, and virus isolations were accomplished by cocultivation of 10^5 to 10^6 PBL on monolayer cultures of indicator sheep choroid plexus cells (*4*). Peripheral blood leukocytes from uninoculated control sheep failed to yield virus.

All four inoculated sheep responded with neutralizing antibody to the inoculum virus within 6 months, but serums failed to neutralize another prototype strain of visna virus, D1-2 (Fig. 1) (*10*). Both virus strains, 1514 and D1-2 were derived from a common ancestral strain K796 (*4*). Four strains of virus isolated from leukocytes of the four sheep were examined by neutralization with isologous and homologous serums. Each leukocyte-derived virus was found to be distinct from the parent strain. Viruses isolated from leukocytes of sheep Nos. 1, 2, and 3 could not be neutralized by immune serums from the three animals but were neutralized by low dilutions of serum from sheep No. 4. The virus recovered from leukocytes of the fourth sheep was neutralized by isologous serum, but this serum neutralized the inoculum virus at higher dilutions (Fig. 1).

The viruses from leukocytes were propagated in vitro for three or more passages at limiting dilutions, and neutralization tests indicated that they had maintained their antigenic identity (*6*). These agents were identified as visna viruses by (i) neutralization by serum from a goat hyperimmunized with both prototype viruses, 1514 and D1-2, and (ii) fluorescent antibody staining with hyperimmune goat serum on cell cultures infected with each of the viruses (*6*). In contrast to postinfection immune sheep serums which tended to have narrow neutralizing activity, hyperimmunization of the goat induced neutralizing antibodies whose activity apparently extended beyond the antigenic determinants of the two immunogens.

Complement fixation (CF) tests, with