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Preparation and Properties of Bovine Factor VIII (Antihemophilic Factor)†

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ABSTRACT: Factor VIII has been purified approximately 300 000-fold from bovine plasma by ammonium sulfate fractionation, glycine precipitation, DEAE-Sephadex column chromatography, sulfate–Sepharose column chromatography, Sephadex G-200 gel filtration, and factor X–Sepharose column chromatography. The highly purified preparation migrated as a triplet on sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis with apparent molecular weights of 93 000, 88 000, and 85 000. The coagulant activity of the purified preparations was inhibited by antibodies raised in rabbits against either the purified factor VIII protein or a preparation of factor VIII/von Willebrand factor. Antibodies to the purified protein also inhibited the coagulant activity of factor VIII/von Willebrand factor preparations. The purified factor VIII contained no platelet-aggregating activity, as measured in human platelet-rich plasma. The purified preparation of factor VIII was required for the activation of factor X in the presence of factor IX_a, calcium, and phospholipid. It was activated about 30-fold by thrombin or factor X_a plus calcium and phospholipid, and each of these reactions was accompanied by a change in the sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis pattern of the protein. Factor VIII was rapidly inactivated by bovine-activated protein C in a reaction requiring calcium and phospholipid. This reaction was also associated with a change in the sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis pattern of the highly purified protein. These experiments involving three highly specific serine proteases support the conclusion that the triplet observed on polyacrylamide gels is factor VIII.

Classic hemophilia and von Willebrand's disease are the two most common disorders of blood coagulation, occurring at a combined frequency of approximately once in 5000 births. Factor VIII (antihemophilic factor)[1] is the coagulant protein which is inactive or absent in individuals with classic hemophilia, a sex-linked clotting disorder affecting males. von Willebrand's disease is inherited in an autosomal manner and affects both male and female. Individuals with this disease lack another plasma protein which is required for platelet aggregation, and this protein has been called von Willebrand factor (vWF). Factor VIII is often low or absent in individuals with von Willebrand's disease, indicating a close association of these two plasma proteins. Accordingly, protein preparations containing coagulant as well as platelet-aggregating activity have been referred to as factor VIII/vWF.

Factor VIII participates in the intrinsic pathway of blood coagulation, where it is involved in the conversion of factor X to factor X_a (Davie & Fujikawa, 1975). The activation of factor X also requires factor IX_a, calcium, and phospholipid (Lundblad & Davie, 1964; Hougie et al., 1967; Hemker & Kahn, 1967; Barton, 1967; Østerud & Rapaport, 1970; Chuang et al., 1972).

Factor VIII and vWF readily copurify, and preparations have been enriched 5000–10 000-fold for coagulant activity from human and bovine plasmas (Schmer et al., 1972; Legaz et al., 1973; Shapiro et al., 1973). These preparations, generally referred to as factor VIII/vWF, show a single, sharp precipitin line upon immunoelectrophoresis and have a molecular weight of greater than one million. Upon reduction, they form subunits with an apparent molecular weight of about 200 000, as measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A number of investigators have reported the dissociation of the coagulant activity from the platelet-aggregating activity in the presence of 1 M sodium chloride or 0.25 M calcium chloride. This has been observed with bovine factor VIII (Thelin & Wagner, 1961; Griggs et al., 1973; Donati et al., 1973), human factor VIII (Weiss & Kochwa, 1970; Weiss et al., 1972; Weiss & Hoyer, 1973; Rick & Hoyer, 1973, 1975), and canine factor VIII (Thelin & Wagner, 1961; Owen & Wagner, 1972; Cooper et al., 1973). Furthermore, the coagulant activity has been separated from

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[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959). Factor VIII (antihemophilic factor) described in this report refers to that protein possessing only coagulant activity. Preparations containing coagulant activity and platelet-aggregating activity are referred to as factor VIII/vWF.

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the platelet-aggregating activity by ion-exchange chromatography (Baugh et al., 1974; Olson et al., 1977) and antigen–antibody chromatography (Holmberg & Ljung, 1978; Horowitz et al., 1979; Tuddenham et al., 1979). These studies have led to the suggestion that factor VIII is composed of a high molecular weight protein with platelet-aggregating activity, and this protein is closely associated with a lower molecular weight subunit which possesses factor VIII coagulant activity. Other investigations, however, have suggested that factor VIII has a covalently linked subunit structure and the molecule has both coagulant and platelet-aggregating activities (Switzer & McKee, 1976).

In the present manuscript, we wish to report the isolation and properties of the coagulant protein from bovine plasma. This preparation is free of platelet-aggregating activity. Furthermore, it is cleaved by limited proteolysis during its activation by factor X_a or thrombin and during its inactivation by activated protein C.

Experimental Section

Materials

Glycine ethyl ester and chlorosulfonic acid were products of MCB, Cincinnati, OH. Dithiothreitol, 2-(*N*-morpholino)ethanesulfonic acid (Mes), imidazole, soybean trypsin inhibitor, 2-mercaptoethanol, Trisma (Tris), bovine serum albumin, poly(ethylene glycol) (PEG-4000), glycine, and ethylenediaminetetraacetic acid were purchased from Sigma Chemical Co., St. Louis, MO. Acrylamide, bis-(acrylamide), and Temed were the products of Bio-Rad Laboratories, Richmond, CA. Lithium heparin was purchased from Riker Laboratories, Inc., Northridge, CA. Cyanogen bromide was a product of Pierce Chemical Co., Rockford, IL. Sephadex G-200, DEAE-Sephadex A-50, Sepharose CL-4B, and the low molecular weight electrophoresis standard were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Schiff reagent was purchased from Scientific Products, McGaw Park, IL. The phospholipid source was Centrolex-P, purchased from Central Soya, Chicago, IL. Bovine brain cephalin, used for the activation of factor X, was prepared according to the method of Bell & Alton (1954). Adjuvants were obtained from Difco Laboratories, Detroit, MI. All other chemicals were of reagent grade.

Factor VIII deficient plasma, factor IX deficient plasma, and factor X deficient plasma were kindly supplied by Dr. Richard Counts of the Puget Sound Blood Center, Seattle, or purchased from George King Bio-medical, Inc., Salem, NH. Factor V deficient plasma was a gift from William Canfield in our laboratory. Bovine factor $X_{a\beta}$ and factor XI_a were kindly provided by Dr. K. Kurachi, and bovine factor IX, activated protein C (Kisiel et al., 1977), factor X, and thrombin were kindly provided by Dr. W. Kisiel in our laboratory. Bovine factor IX_a was prepared by incubating factor IX with factor XI_a (40:1 weight ratio) in the presence of 5 mM calcium chloride (Fujikawa et al., 1974a). Factor IX_a was then separated from factor XI_a by chromatography on a DEAE-Sephadex column. Factor VIII/vWF was prepared by the method of Legaz & Davie (1976).

Methods

Protein concentrations were determined by adsorption at 280 nm assuming an $E_{280}^{1\%}$ of 10.0 for factor VIII, 19.5 for thrombin, 14.3 for factor IX_a, 11.5 for factor X, 10.0 for factor $X_{a\beta}$, 13.7 for activated protein C, and 12.6 for factor XI_a. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the following modification of the method of Swank & Munkres (1971). Samples were dialyzed against 0.01 M phosphate, pH 7.0, containing 1% sodium dodecyl sulfate and 8 M urea, and boiled for 2 min prior to electrophoresis. Reduced samples were prepared in the presence of 5% 2-mercaptoethanol. Polyacrylamide gels contained 5% acrylamide, 8 M urea, and 0.10 M H_3PO_4 adjusted to pH 7.0 with Tris. The reservoir buffer contained 0.1 M H_3PO_4, adjusted to pH 7.0 with Tris, and 0.1% sodium dodecyl sulfate. Samples were subjected to electrophoresis at 4 mA/gel until the tracking dye (bromophenol blue) was 5 mm from the end of the tube (approximately 6 h). Gels were stained for protein for 1 h with a solution of 45% methanol, 9% acetic acid, and 0.25% Coomassie brilliant blue R250 and destained with 7.5% methanol and 5% acetic acid in a Bio-Rad gel electrophoresis diffusion destainer. Molecular weights were obtained by interpolation from a linear semilogarithmic plot of apparent molecular weight vs. migration distance by using a Pharmacia low molecular weight electrophoresis standard.

Factor VIII was assayed in factor VIII deficient plasma by the procedure of Legaz & Davie (1976). Assays with von Willebrand plasma and factor IX deficient plasma followed the same procedure. Factor V activity was assayed by the method of Kappeler (1955). Citrated plasma, pooled from five animals and stored at −20 °C, was used as a reference standard. One unit of activity is defined as that activity present in 1 mL of pooled citrated bovine plasma.

Platelet aggregation was monitored by using a Payton dual channel aggregation module. Assays were performed by prewarming 0.25 mL of platelet-rich human plasma at 37 °C for 2 min while stirring at 600 rpm. Protein samples (10 μL or less) were then added and the decrease in light transmission was followed.

Sulfate–Sepharose was prepared as follows. Sepharose CL-4B (75-mL settled volume) was washed extensively with acetone to remove water, and the resin was then equilibrated with formamide and suspended in a final volume of 120 mL. A solution of 4 mL of chlorosulfonic acid dissolved in 10 mL of dichloromethane was added, with stirring, over a 2-min period at 22 °C. Stirring was then continued for 1 h. The resin was added to 4 L of water, and the pH was rapidly adjusted to neutrality with concentrated sodium hydroxide.

Proteins were coupled to Sepharose by the procedure of Porath (1974). Sepharose CL-4B was washed on a Millipore funnel with 2 M potassium phosphate, pH 12.0, and suspended in a total volume of 4 mL/g of washed resin. Cyanogen bromide (40 mg/g of resin) was dissolved in formamide (2.5 mL/g of cyanogen bromide) and slowly added to the resin while stirring for 2 min. The mixture was then stirred for an additional 8 min and the temperature was maintained at approximately 10 °C by using an ice bath. The slurry was quickly filtered under vacuum and washed with ice-cold water followed by ice-cold 0.25 M sodium carbonate, pH 8.4. The resin was rapidly transferred to a beaker, and a solution of protein to be coupled (approximately 1–4 mg/g of resin) in 0.25 M sodium carbonate, pH 8.4, was added. The suspension was stirred slowly for 16 h at 4 °C, filtered, and stored at 4 °C as a slurry in 0.02 M imidazole, pH 6.8, containing 0.15 M NaCl.

Antibodies were raised in New Zealand white rabbits following the procedure of Harboe & Ingild (1973). Factor VIII/vWF antibodies were raised by using Freund's complete adjuvant with the first injection. The antibodies were isolated from sera by repeated precipitation with ammonium sulfate added to 40% saturation. The antibodies were dialyzed against 0.07 M sodium acetate, pH 5.0, and chromatographed on a DEAE-Sephadex resin previously equilibrated with the same

Table I: Purification of Bovine Factor VIII

purifn step	vol (mL)	total protein[a] (mg)	total act. (units)	sp act. (units/mg)	recovery (%)	purifn (x-fold)
plasma	125 000	8.8×10^6	(125 000)	0.014	(100)	(1)
$(NH_4)_2SO_4$ fractionation	10 500	4.8×10^5	31 000	0.06	25	4.3
glycine precipn	10 500	4.3×10^5	29 000	0.07	24	4.7
second glycine precipn and $(NH_4)_2SO_4$ precipn	450	13 000	21 100	1.6	17	114
DEAE-Sephadex	125	94	16 000	165	13	12 000
sulfate-Sepharose	7	4.9	10 000	2100	8	150 000
Sephadex G-200	36	1.2	1 600	1333	1	95 000
factor X-Sepharose	2	0.4	1 800	4500	1.4	320 000

[a] Protein concentration was determined by absorption assuming $E_{280}^{1\%} = 10.0$.

buffer (Harboe & Ingild, 1973). The nonabsorbed fractions containing the antibody were pooled, concentrated to approximately 30 mg/mL, and stored at −20 °C.

Purification of Factor VIII. Bovine blood was collected from the slaughterhouse and rapidly mixed with 0.1 volume of an anticoagulant solution consisting of 0.1 M trisodium citrate, heparin (13 000 units/L), and crude soybean trypsin inhibitor (160 mg/L). Plasma was prepared at room temperature utilizing a continuous flow separator (DeLaval Model BLE 519). Subsequent steps were performed at 4 °C unless otherwise indicated. Barium chloride (80 mL of 1.0 M solution per L of plasma) was added and the resulting suspension was stirred for 45 min. The barium citrate precipitate was removed by centrifugation at 6500*g* for 10 min in a Sorvall RC3 centrifuge. Solid ammonium sulfate was added to the supernatant to 20% saturation, and the solution was stirred for 40 min and centrifuged as described above. The precipitate was dissolved by stirring for 1 h at room temperature in one-tenth the original plasma volume in 0.02 M imidazole hydrochloride buffer, pH 6.8, containing 0.2 M sodium chloride. Insoluble material was removed by centrifugation for 10 min as described above. Glycine was added to the supernatant to a concentration of 2.1 M, and the precipitate was collected by centrifugation for 5 min as described above. The protein precipitate was then frozen at −20 °C.

The frozen protein precipitate from 125 L of plasma was thawed and dissolved in a total volume of 10.5 L of 0.02 M imidazole hydrochloride buffer, pH 6.8, containing 0.2 M sodium chloride. Glycine ethyl ester and calcium chloride were added to final concentrations of 0.05 and 0.02 M, respectively. The solution was made 30 mM with 2-mercaptoethanol and stirred at 4 °C for 15 h. Glycine was again added to 2.1 M, and the solution was stirred for 20 min. The precipitate was removed by centrifugation for 10 min at 6500*g*, and solid ammonium sulfate was then added to the supernatant to obtain 35% saturation. The solution was stirred for 40 min and centrifuged for 20 min at 6500*g* at 4 °C. The precipitate was suspended in 200 mL of 0.02 M imidazole hydrochloride, pH 6.6, containing 0.15 M sodium chloride, 0.01 M calcium chloride, 0.02 M glycine ethyl ester, 10% glycerol, and 0.5 mM dithiothreitol, and dialyzed for 24 h against 6 L of the same buffer. This buffer, used throughout the remaining purification procedure, has been referred to as buffer A, with the concentration of sodium chloride specified. The protein sample was applied to a 5 × 50 cm DEAE-Sephadex A-50 column equilibrated with the same buffer. The resin was washed with 2 L of this buffer and eluted with a gradient of 1 L of buffer A containing 0.15 M sodium chloride and 1 L of 0.02 M imidazole hydrochloride, pH 6.3, containing 0.01 M 2-(*N*-morpholino)ethanesulfonic acid, 1.5 M sodium chloride, 0.2 M glycine ethyl ester, 0.1 M calcium chloride, 10% glycerol, and 0.5 mM dithiothreitol. Factor VIII eluted as a broad protein peak. Fractions with coagulant activity were pooled and the solution was made 15% with poly(ethylene glycol)-4000. After being allowed to stand for 2 h at 0 °C, the precipitate was removed by centrifugation for 10 min at 3000*g* in a Sorvall RC5 centrifuge. The protein precipitate was then dissolved by stirring for 3 h in 120 mL of buffer A containing 0.15 M sodium chloride. Insoluble material was removed by centrifugation for 10 min at 8000*g* at 4 °C and discarded. The supernatant was adsorbed on a 0.9 × 6 cm sulfate–Sepharose column previously equilibrated with buffer A containing 0.15 M sodium chloride. When the absorbance at 280 nm dropped below 0.01, coagulant activity was eluted with buffer A containing 0.65 M sodium chloride. Fractions containing coagulant activity were pooled and concentrated with an Amicon Diaflo chamber using a PM-10 membrane. The sample was then chromatographed on a 2.6 × 95 cm Sephadex G-200 column previously equilibrated with buffer A containing 0.15 M sodium chloride. The eluted fractions containing factor VIII activity were pooled and 0.2 volume of water was added to lower the ionic strength. The sample was applied to a 0.9 × 3.5 cm factor X–Sepharose column previously equilibrated with 0.02 M imidazole hydrochloride buffer, pH 6.6, containing 0.08 M calcium chloride, 0.017 M glycine ethyl ester, 10% glycerol, and 0.5 mM dithiothreitol. Elution from this column was accomplished with buffer A containing 0.5 M sodium chloride. Fractions of 2.6 mL were collected during the application of the sample and washing of this column. Fractions of 0.4 mL were then collected during elution. The fractions with factor VIII activity were pooled and dialyzed for 4–6 h against buffer A containing 0.15 M sodium chloride.

The highly purified factor VIII was stored at 0 °C and employed without freezing during the next 24 h for various studies. Freezing in buffer A at −20 °C for up to 1 month resulted in a loss of about 30% of the coagulant activity.

Results

Purification of Bovine Factor VIII. Factor VIII was purified approximately 300 000-fold from bovine plasma with an overall yield of about 1% (Table I). Factor VIII activity could not be assayed in the starting plasma which contained high levels of inhibitors. Therefore, citrated plasma collected in the absence of protease inhibitors was employed as a plasma standard. The purification procedure includes ammonium sulfate fractionation, glycine precipitation, DEAE-Sephadex column chromatography, sulfate–Sepharose column chromatography, Sephadex G-200 gel filtration, and factor X–Sepharose column chromatography.

Ammonium sulfate fractionation was employed as the first step in the purification procedure in order to reduce the volume more than 10-fold. Only 25% of the original factor VIII coagulant activity in plasma was recovered in the ammonium sulfate precipitation step and attempts to improve the recovery

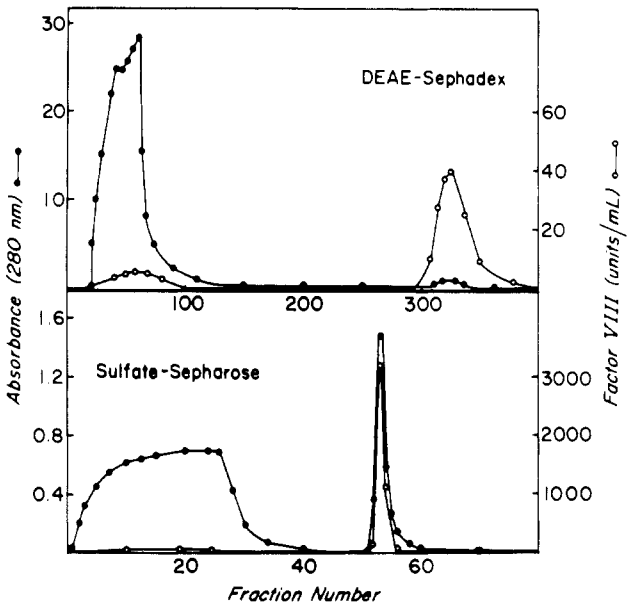

FIGURE 1: (Top panel) Elution pattern for chromatography of factor VIII on a DEAE-Sephadex A-50 column (5 × 50 cm). Factor VIII (450 mL) was applied to the column which was then washed with 2 L of 0.02 M imidazole hydrochloride, pH 6.6, containing 0.15 M sodium chloride, 0.01 M calcium chloride, 0.02 M glycine ethyl ester, 10% glycerol, and 0.5 mM dithiothreitol. Factor VIII was eluted with a linear gradient formed from 1 L of the above buffer and 1 L of 0.02 M imidazole hydrochloride, 0.01 M 2-(*N*-morpholino)ethanesulfonic acid, 1.5 M sodium chloride, 0.1 M calcium chloride, 0.2 M glycine ethyl ester, 10% glycerol, and 0.5 mM dithiothreitol, pH 6.3. The gradient was started at fraction 250. Fractions (11 mL) were collected at a flow rate of 70 mL/h. (Lower panel) Elution pattern for chromatography of factor VIII on a sulfate–Sepharose column (0.9 × 6 cm). The nonadsorbed protein was washed from the column with 0.02 M imidazole hydrochloride, pH 6.6, containing 0.15 M sodium chloride, 0.01 M calcium chloride, 0.02 M glycine ethyl ester, 10% glycerol, and 0.5 mM dithiothreitol. Factor VIII was eluted by the above buffer containing 0.65 M sodium chloride. Five-milliliter samples were collected through fraction 50; fractions 51–60 were collected in 2.0-mL samples. The flow rate was 60 mL/h. (●) Absorbance at 280 nm; (○) factor VIII coagulant activity.

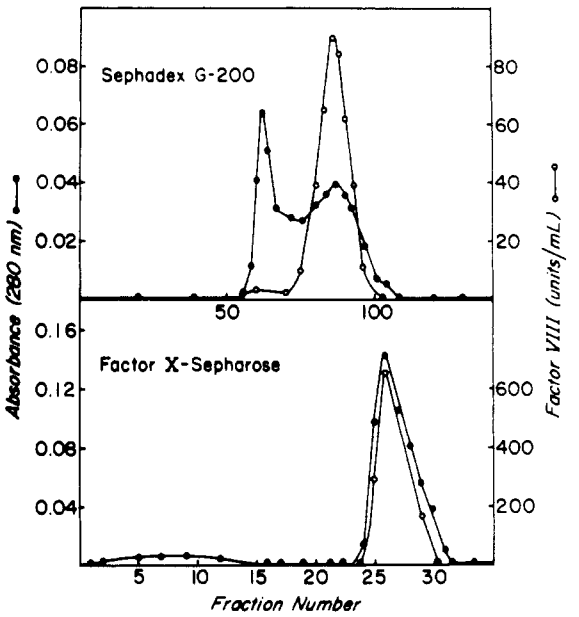

FIGURE 2: (Top panel) Elution pattern for the gel filtration of factor VIII on a Sephadex G-200 column (2.6 × 95 cm). The protein from the sulfate–Sepharose column was concentrated to 2.0 mL and applied to the column. Protein was eluted with 0.02 M imidazole hydrochloride, pH 6.6, containing 0.15 M sodium chloride, 0.02 M glycine ethyl ester, 10% glycerol, and 0.5 mM dithiothreitol. Fractions (2.6 mL) were collected at a flow rate of 11 mL/h. (Lower panel) Elution pattern for factor VIII from a factor X–Sepharose column (0.9 × 3.5 cm). Factor VIII was pooled from the Sephadex G-200 column and diluted with 0.2 volume of water. The sample was then applied to the factor X column previously equilibrated with 0.02 M imidazole hydrochloride, pH 6.6, containing 0.125 M sodium chloride, 0.08 M calcium chloride, 0.017 M glycine ethyl ester, 10% glycerol, and 0.5 mM dithiothreitol. The flow rate was 40 mL/h. After the column was washed with 26 mL of the above buffer, the protein was eluted with 0.02 M imidazole hydrochloride, pH 6.6, containing 0.5 M sodium chloride, 0.01 M calcium chloride, 0.02 M glycine ethyl ester, 10% glycerol, and 0.5 mM dithiothreitol. Fractions of 2.6 mL were collected during the sample application and column washing, while fractions of 0.4 mL were collected during the elution. (●) Absorbance at 280 nm; (○) factor VIII activity.

in this step have been unsuccessful thus far.

Factor VIII and fibrinogen were precipitated by the first glycine precipitation step, while most of the cold insoluble globulin remained in the supernatant fraction. Fibrinogen was removed by the second glycine precipitation after the addition of 30 mM 2-mercaptoethanol (or 4 mM dithiothreitol). Factor VIII activity remained in the supernatant fraction during this procedure. Ammonium sulfate precipitation then resulted in a factor VIII preparation concentrated about 300-fold from the starting plasma with a purification of over 100-fold.

Factor VIII was purified another 100-fold by chromatography on DEAE-Sephadex (top panel, Figure 1). Recovery on the DEAE-Sephadex column was greater than 70%. Factor VIII was then fractionated on a sulfate–Sepharose column where the activity was eluted by a stepwise increase in the salt concentration of the buffer (lower panel, Figure 1). The purification in this step was consistently greater than 10-fold with a recovery of about 60% (Table I).

Gel filtration on Sephadex G-200 was then employed in the isolation procedure. Cold insoluble globulin was readily bound to the factor X–Sepharose column where it eluted with factor VIII. Accordingly, it was essential to remove this protein and other high molecular weight contaminants prior to the last step of the isolation procedure. Factor VIII eluted with an apparent molecular weight of 250 000–300 000 from the Sephadex G-200 column (top panel, Figure 2). Attempts to improve the recovery of activity during gel filtration by changing the ionic strength, the addition of protease inhibitors, or pooling fractions have been unsuccessful. Whether the loss of activity is due to an inactivation of the protein or to the removal of an activated form(s) of factor VIII is not known.

The final step in the purification of factor VIII was an affinity chromatography step employing factor X–Sepharose (lower panel, Figure 2). This procedure removed small amounts of contaminants and also concentrated the protein from the Sephadex G-200 column.

Sodium Dodecyl Sulfate/Urea–Polyacrylamide Gel Electrophoresis. The highly purified factor VIII migrated as a triplet in sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis in the presence or absence of 2-mercaptoethanol (Figure 3, gels 1, 3, and 6). The molecular weights of the factor VIII triplet estimated by this technique were approximately 85 000, 88 000, and 93 000 for the reduced samples. Evidence that these bands are probably associated with the factor VIII coagulant protein is presented below. In the absence of denaturing conditions, the highly purified preparation of factor VIII does not enter the polyacrylamide gel. This indicates that the purified preparation readily forms aggregates, and this could explain the apparent molecular weight of 250 000–300 000 observed by gel filtration on Sephadex G-200.

Clotting Activity of the Highly Purified Factor VIII. Purified factor VIII readily corrected the clotting time of he-

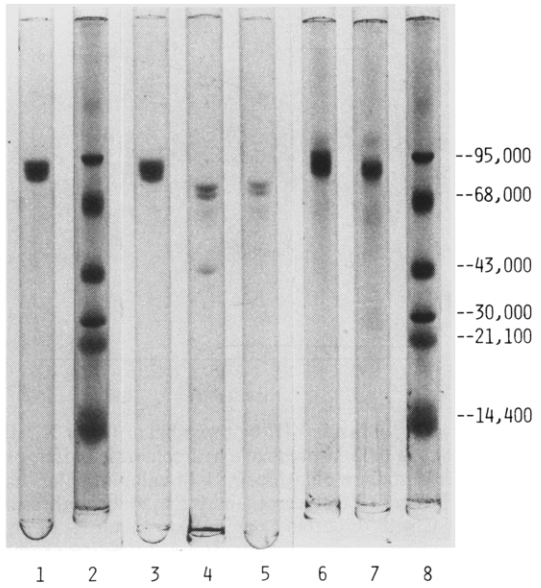

FIGURE 3: Sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis of factor VIII. Gels 1, 3, and 6 are factor VIII; gels 2 and 8 are protein standards; gel 4 is thrombin-activated factor VIII; gel 5 is thrombin-activated factor VIII incubated with activated protein C; gel 7 is factor VIII incubated with activated protein C. Each gel contained 40–50 μg of factor VIII. For thrombin activation, factor VIII (45 μg) in 0.02 M imidazole hydrochloride buffer, pH 6.6, containing 0.15 M sodium chloride, 0.01 M calcium chloride, 0.02 M glycine ethyl ester, 10% glycerol, and 0.5 mM dithiothreitol was incubated with thrombin (1 μg) for 20 min. For inactivation by activated protein C, factor VIII was activated by thrombin as described above and then incubated for 20 min with activated protein C (1 μg) and phospholipid (100 μg/mL Centrolex-P). For the inactivation of factor VIII by activated protein C, 45 μg of factor VIII was incubated for 20 min with activated protein C (1 μg) and phospholipid (100 μg/mL Centrolex-P). All incubations were at 20 °C. The protein standards (gels 2 and 8) consisted of phosphorylase *b* (95 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (21 100), and α-lactalbumin (14 400).

Table II: Effect of Factor VIII on the Coagulant Activity of Various Deficient Plasmas[a]

substrate	act. (units/mL)[b]
human factor VIII deficient plasma	1210
human von Willebrand deficient plasma	830
human factor IX deficient plasma	9
bovine factor X deficient plasma	0
human factor V deficient plasma	0.1

[a] Assay conditions are described under Methods. [b] Factor VIII solution was 0.3 mg/mL and was diluted 1:10 to 1:10 000 for the assays.

mophilic plasma and von Willebrand plasma (Table II). The highly purified preparation had essentially no effect on the clotting time of factor X deficient plasma or factor V deficient plasma. A very small correction of factor IX deficient plasma, however, was observed.

Inhibition of Factor VIII Coagulant Activity by Antibodies against the Highly Purified Protein or Factor VIII/vWF. The factor VIII coagulant activity of bovine citrated plasma was strongly inhibited by antibodies raised in rabbits against either highly purified factor VIII or the factor VIII/vWF preparation (top panel, Figure 4). Furthermore, the coagulant activity of highly purified factor VIII was inhibited by antibody against highly purified factor VIII or factor VIII/vWF (lower panel, Figure 4). In addition, the concentration of antibody against purified factor VIII that was required for 50% inhibition of the coagulant activity of purified factor VIII (1 unit/mL) also

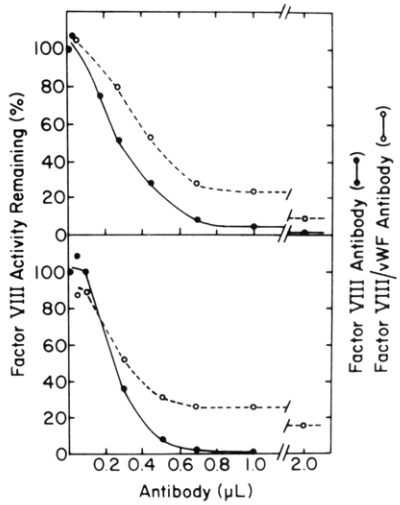

FIGURE 4: Effect of antibody concentration on factor VIII coagulant activity. (Top panel) Inhibition of factor VIII coagulant activity from plasma. Antibodies were added to individual samples of 0.2 mL of plasma at the concentration indicated. The samples were incubated at 20 °C for 1 h and assayed for coagulant activity. (Lower panel) Inhibition of purified factor VIII. Factor VIII was diluted in 0.02 M imidazole hydrochloride buffer, pH 6.6, containing 0.15 M sodium chloride, 0.01 M calcium chloride, 0.02 M glycine ethyl ester, 10% glycerol, 0.5 mM dithiothreitol, and 1 mg/mL bovine serum albumin. Factor VIII activity was 1 unit/mL upon a 1:10 dilution. Antibodies were added to individual samples at 20 °C for 20 min and assayed for factor VIII levels. All samples were diluted 1:10 for assay of factor VIII coagulant levels. The antibody concentration was 30 μg/mL.

resulted in a 50% inhibition of the factor VIII coagulant activity in plasma (1 unit/mL). This indicates that the protein in whole plasma which is responsible for factor VIII coagulant activity has essentially the same antigenic properties as the highly purified preparation which we have isolated. This suggests that the highly purified protein is very similar, and possibly identical, to that in whole plasma.

Platelet Aggregation. Studies on platelet aggregation were performed with human platelet-rich plasma to compare the highly purified bovine factor VIII coagulant protein with the bovine factor VIII/vWF preparation which contained both coagulant and platelet-aggregating activity. The addition of purified bovine factor VIII to human platelet-rich plasma resulted in no detectable platelet-aggregating activity (arrow 1, top panel, Figure 5). The addition, however, of a bovine factor VIII/vWF preparation 100 times less concentrated on the basis of coagulant activity resulted in rapid platelet aggregation (arrow 2, top panel). These data indicate that the highly purified preparation of factor VIII is free of platelet-aggregating activity. It is possible, however, that platelet-aggregating activity in the highly purified preparation was destroyed by the presence of dithiothreitol which was added throughout the purification procedure. The destruction of this activity by reducing agents has been reported earlier by others (Cooper et al., 1975; Brown et al., 1976).

It has been suggested that a coagulant subunit of factor VIII in the factor VIII/vWF preparation might arise by processing of the 200 000-dalton subunit (Austen, 1976; Switzer & McKee, 1976, 1977). This proposal was tested in the following experiments. The addition of bovine plasma as a source of factor VIII/vWF readily resulted in the aggregation of human platelet-rich plasma (curve 1, lower panel, Figure 5). The addition of antibodies against the purified factor VIII had no detectable effect on this platelet aggregation initiated by bovine plasma (curve 2, lower panel). In contrast, the addition of antibodies against factor VIII/vWF in an amount 4 times less

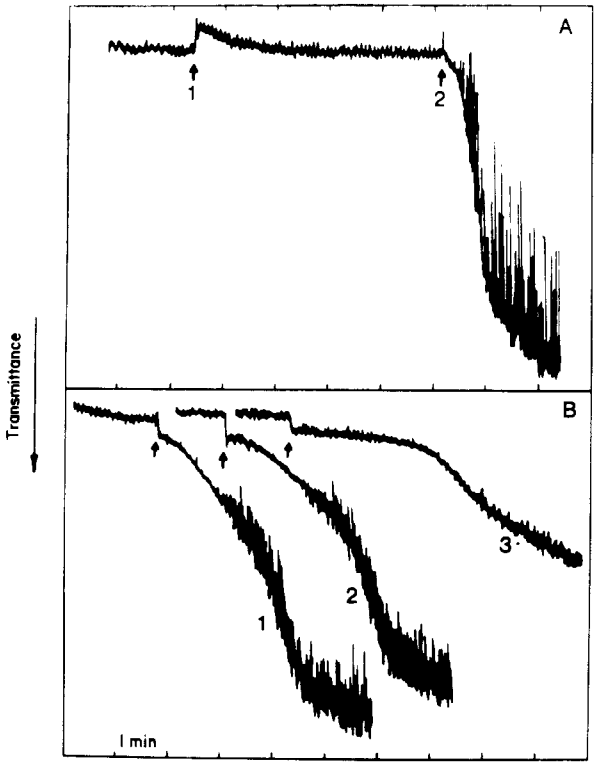

FIGURE 5: Aggregation of human platelets with bovine factor VIII/vWF. (Top panel) Effect of purified factor VIII and factor VIII/vWF on human platelets. Purified factor VIII (10 μL of 0.14 mg/mL; 600 units/mL) was added as indicated by arrow 1; factor VIII/vWF (10 μL of 0.35 mg/mL; 6 units/mL) was added as indicated by arrow 2. (Lower panel) Curve 1: 5 μL of citrated bovine plasma containing factor VIII/vWF added at the time indicated by the arrow. Curve 2: 5 μL of citrated bovine plasma containing purified factor VIII antibody added at the time indicated by the arrow. Curve 3: 5 μL of citrated bovine plasma containing factor VIII/vWF antibody added at the time indicated by the arrow. Bovine plasma containing antibody was prepared by incubating 1.0 mL of bovine plasma with 60 μL of purified factor VIII antibody or 15 μL of factor VIII/vWF antibody for 2 h at 20 °C. Platelet aggregation was monitored as described under Methods.

(based on coagulant activity) than that employed for the experiment shown in curve 2 resulted in a substantial inhibition of the platelet-aggregating activity of the bovine plasma (curve 3, lower panel). If the coagulant portion of factor VIII in the factor VIII/vWF preparation arises from the 200 000-dalton subunit, one would expect some of the antigenic determinants would be shared by the two proteins. The fact that antibodies to highly purified factor VIII do not affect platelet aggregation is consistent with the concept that the coagulant protein is a separate entity in the factor VIII/vWF activity in whole plasma and probably does not arise from the 200 000-dalton subunit.

Activation of Factor VIII by Thrombin and Factor X_a. Thrombin has been reported to increase the coagulant activity of factor VIII (Rapaport et al., 1963, 1965; Macfarlane et al., 1964; Ozge-Anwar et al., 1965; Legaz et al., 1973, 1975; Shapiro et al., 1973). Factor X_a has also been shown to result in the activation of factor VIII (Davie et al., 1975). A time curve for the activation of purified factor VIII by thrombin is shown in Figure 6 (solid circles). Factor VIII coagulant activity increased about 30-fold with a weight ratio of factor VIII to thrombin of 260:1. The activation proceeded rapidly at pH 6.6 with a stable coagulant activity being formed. Increasing the pH of the reaction mixture to 7.0, or the addition of 0.02 M EDTA, resulted in a rapid loss of activity of the activated form. Factor VIII was also activated by factor X_a

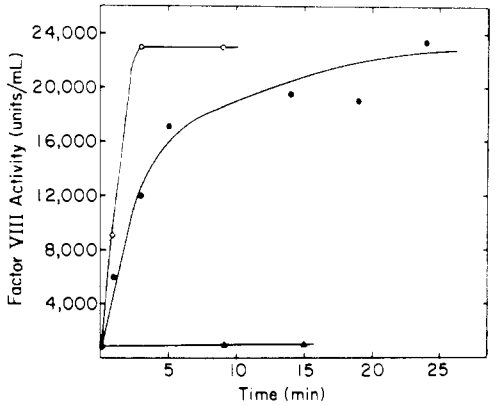

FIGURE 6: Activation of factor VIII by thrombin or factor X_a. Factor VIII (260 μg/mL) in 0.02 M imidazole hydrochloride buffer, pH 6.6, containing 0.15 M sodium chloride, 0.01 M calcium chloride, 0.02 M glycine ethyl ester, 10% glycerol, and 0.5 mM dithiothreitol was activated at 20 °C with thrombin (1 μg/mL) or factor X_a (2 μg/mL) with or without phospholipid (100 μg/mL). At the times indicated, samples were removed and assayed for factor VIII activity. (●) Thrombin; (○) factor X_a plus phospholipid; (▲) factor X_a.

in the presence of calcium and phospholipid (open circles). In these experiments, the weight ratio of factor VIII to factor X_a was 130:1. In the absence of phospholipid, essentially no activation of factor VIII occurred in the presence of factor X_a (solid triangles).

A proteolytic cleavage of factor VIII associated with thrombin activation was demonstrated by sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis (Figure 3). Following thrombin activation, the factor VIII triplet (gel 3) was converted to a doublet with molecular weights of 69 000 and 73 000, a faint, diffuse-staining band of about 55 000 molecular weight, and a band of 38 000 molecular weight (gel 4). Higher concentrations of thrombin (enzyme to substrate ratio of 1:10) gave the same polyacrylamide gel electrophoresis pattern. The activation of factor VIII by factor X_a in the presence of calcium and phospholipid gave a sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis pattern which was indistinguishable from that obtained with thrombin. These results are consistent with the concept that the triplet observed in gel 3 is factor VIII and that the activation of this clotting factor by thrombin or factor X_a is due to limited proteolysis.

Activation of Factor X. In previous experiments, factor VIII has been shown to be required for the conversion of factor X to factor X_a in the presence of factor IX_a, calcium, and phospholipid (Lundblad & Davie, 1964; Hougie et al., 1967; Hemker & Kahn, 1967; Barton, 1967; Østerud & Rapaport, 1970; Chuang et al., 1972; Fujikawa et al., 1974b). A similar requirement was also noted for the purified factor VIII in the present experiments in which the initial rates for factor X_a formation were examined (Figure 7). The activation of factor X in this system occurred after a 2–3-min lag phase (solid circles). Similar effects have been previously reported (Fujikawa et al., 1974b; Brown et al., 1978; Hultin & Nemerson, 1978). The lag phase, however, was greatly reduced upon the addition of factor X_a (open triangles) or thrombin (open circles). In the absence of factor VIII or calcium or phospholipid, no activation of factor X occurred (solid triangles). Preincubation of factor VIII with factor IX_a, calcium, and phospholipid for 2 min prior to the addition of the factor X completely eliminated the lag phase (open squares). This reaction was carried out in the absence of thrombin or factor X_a. It appears to involve a different mechanism than that observed with thrombin or factor X_a since factor IX_a does not enhance the coagulant activity of factor VIII in experiments

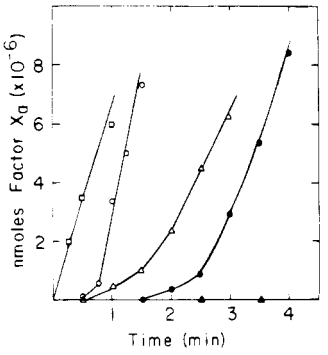

FIGURE 7: Activation of factor X in the presence of factor VIII, factor IX_a, calcium, and phospholipid. The standard reaction mixture contained 0.8 mL of 0.02 M imidazole hydrochloride buffer, pH 7.2, 0.15 M sodium chloride, 0.008 M calcium chloride, 5 μL of cephalin (120 μg/mL), 1 μL of factor IX_a (0.12 mg/mL), 10 μL of factor X (1.4 mg/mL), and 50 μL of S-2222 (1 mM/mL). The activation reaction was initiated by the addition of 1 μL of factor VIII (0.12 mg/mL). Thrombin (1 μg) or factor $X_{a\beta}$ (1 μg) was added to the reaction mixture prior to the addition of factor VIII. For preincubation of factor VIII and factor IX_a, the reaction was initiated, after 2 min of incubation at 30 °C, by the addition of factor X. Due to the rapid activation of factor X, the amounts of factor IX_a and factor VIII were reduced by half in these experiments. The change in absorbance at 405 nm was monitored on a spectrophotometer maintained at 30 °C with a circulating water bath. The change in absorbance per minute was determined at various time points on the activation curve. Conversion of the change in absorbance per minute to nanomoles of factor X activated per minute was made by comparison to a standard curve prepared by the activation of factor X with RVV-X (Kisiel et al., 1976b). (●) Standard reaction mixture; (△) standard reaction mixture plus factor X_a; (○) standard reaction mixture plus thrombin; (□) standard reaction mixture after preincubation of factor VIII and factor IX_a; (▲) standard reaction mixture minus factor VIII or minus calcium or phospholipid.

Table III: Effect of Activated Protein C on the Coagulant Activity of Factor VIII/vWF[a]

experiment	act. (%)
factor VIII/vWF	100
factor VIII/vWF plus activated protein C (21 000:1)	41
factor VIII/vWF plus activated protein C (2100:1)	19
factor VIII/vWF plus DIP-activated protein C[b] (2100:1)	83
factor VIII/vWF plus EDTA[c] plus activated protein C (2100:1)	72

[a] Reaction mixtures contained 0.26 mg of factor VIII/vWF in 0.2 mL of 0.02 M imidazole hydrochloride buffer, pH 7.4, 0.15 M sodium chloride, and 0.005 M calcium chloride. Activated protein C (1 μL) was added at the weight ratio indicated (shown in parentheses). Samples were assayed for factor VIII coagulant activity after incubation for 9 min at 37 °C. [b] DIP-activated protein C (diisopropyl phosphoryl activated protein C) was prepared by incubating the enzyme with 15 mM diisopropyl fluorophosphate for 1 h, followed by dialysis against 0.15 M sodium chloride with 0.02 M imidazole hydrochloride, pH 6.8. [c] EDTA (final concentration of 20 mM) was added prior to the addition of activated protein C.

analogous to those shown in Figure 6.

Effect of Activated Protein C on Factor VIII/vWF and Purified Factor VIII. Bovine activated protein C is a serine protease that markedly prolongs the kaolin–cephalin clotting time of bovine plasma (Kisiel et al., 1976a; Esmon et al., 1976; Kisiel et al., 1977). This effect was shown to be due, in part, to the inactivation of factor V (Kisiel et al., 1977). In the present studies, it was found that very low concentrations of activated protein C (10–100 ng/0.2 mL) readily inactivated the coagulant activity of factor VIII/vWF (Table III). This effect was not dependent upon the addition of phospholipid, in contrast to the inactivation of factor V by activated protein C. The loss of coagulant activity in the presence of activated protein C was observed only when factor VIII/vWF was

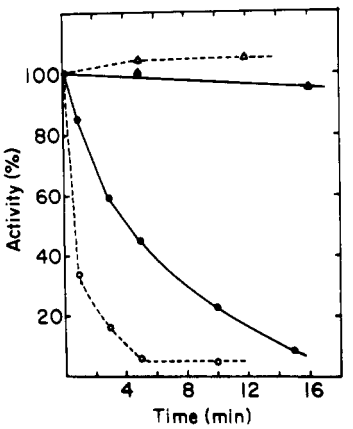

FIGURE 8: Inactivation of purified factor VIII by activated protein C. Factor VIII or thrombin-activated factor VIII (30 μg) was incubated with 0.2 μg of activated protein C in the presence of 0.02 M imidazole hydrochloride buffer, pH 7.0, containing 0.15 M sodium chloride, 0.01 M calcium chloride, 0.02 M glycine ethyl ester, 10% glycerol, 0.5 mM dithiothreitol, and 20 μg of phospholipid in a final volume of 0.15 mL. At the times indicated, aliquots were removed and assayed for factor VIII coagulant activity. Thrombin-activated factor VIII in the presence (○) or absence (△) of activated protein C; factor VIII in the presence (●) or absence (▲) of activated protein C.

preincubated with calcium chloride for several hours prior to the addition of activated protein C. The reason for this effect is not known. Complexing the calcium by the addition of EDTA prevented most of the loss of coagulant activity (Table III).

Thrombin-activated factor VIII/vWF, stabilized in the presence of 0.25 M calcium chloride (Vehar & Davie, 1977), was also rapidly inhibited by activated protein C. Pretreatment of activated protein C with 0.015 M diisopropyl fluorophosphate blocked the inactivation reaction (Table III). No cleavage of the 200 000 molecular weight subunit of factor VIII/vWF could be detected by sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis following the inactivation reaction. Furthermore, activated protein C had no effect on the platelet-aggregating activity of bovine factor VIII/vWF in human platelet-rich plasma.

Activated protein C also caused a rapid loss of the coagulant activity of purified factor VIII and the thrombin-activated form of purified factor VIII (Figure 8). The rate of inactivation of the thrombin-activated form of factor VIII (open circles) was significantly faster than the unactivated form (solid circles). The inactivation of purified factor VIII by activated protein C required the presence of calcium and phospholipid, in contrast to the inactivation of factor VIII/vWF by activated protein C.

The proteolytic cleavage of the thrombin-activated factor VIII by activated protein C was demonstrated by sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis (gels 4 and 5, Figure 3). In these experiments, the loss of coagulant activity in the presence of activated protein C was associated with the disappearance of the 38 000 molecular weight protein band. In some experiments, the appearance of a new, faint protein band with a molecular weight of approximately 29 000 was detected.

The loss of coagulant activity of purified factor VIII (gel 6, Figure 3) by activated protein C was associated with the disappearance of the 93 000 molecular weight band of factor VIII (gel 7). The two faster moving bands of the triplet were not affected. No other degradation products were observed.

Factor IX_a in the presence of calcium and phospholipid had no effect on the sodium dodecyl sulfate/urea–polyacrylamide

gel electrophoresis pattern of factor VIII upon incubation at 37 °C for 1 h at a weight ratio of enzyme to substrate of 1:50.

Discussion

Thelin & Wagner (1961) reported the separation of the coagulant activity of factor VIII from an apparent high molecular weight form. Since that time, controversy over the nature, and even the existence, of this protein has been extensive [for recent reviews, see Bloom & Peake (1977) and Austen (1979)]. There is now general agreement that most of the current procedures for isolation of human factor VIII/vWF result in a protein preparation with a molecular weight in excess of one million (Ratnoff et al., 1969; Hershgold et al., 1971; Legaz et al., 1973). This preparation has four closely related properties: (1) factor VIII coagulant activity, (2) an antigen to the factor VIII coagulant activity [presumably identical with (1)], (3) a protein which can cause platelet aggregation, called von Willebrand factor, and (4) an antigen readily precipitated by antisera raised in rabbits against factor VIII/vWF. Resolving the relationship of these activities is essential in understanding the cause(s) of the diseases resulting from a lack of the factor VIII coagulant activity.

The purpose of the present research was to isolate and characterize the protein(s) responsible only for correcting the coagulation defect in factor VIII deficient plasma. This goal has been achieved in that the highly purified factor VIII isolated from bovine plasma has strong coagulant activity but no platelet-aggregating activity in human platelet-rich plasma. The overall yield of the coagulant activity, however, was low and improvements in the isolation procedure are necessary. The low yield at the gel filtration step was particularly troublesome. Factor VIII will bind to many other resins, such as benzamidine–agarose, octyl-Sepharose, heparin–agarose, and dextran sulfate–agarose. Employing various combinations of these resins in place of the gel filtration step, however, has not been successful thus far.

The low yield may in part reflect the difficulties in assaying factor VIII using factor VIII deficient plasma. For instance, plasma samples collected in the presence of high concentrations of protease inhibitors appear to have very low levels of factor VIII activity. This apparent low activity is not due to a carry-over of inhibitors into the assay plasma. This suggests that factor VIII determined in plasma samples collected in citrate in the absence of protease inhibitors may contain some partially activated factor VIII which results in an artificially high estimate of the level of the clotting factor. Accordingly, small amounts of activated factor VIII in the starting plasma or at some step in the isolation procedure will greatly influence the apparent recovery of factor VIII and the extent of purification as reported in Table I.

Bovine blood was employed as the starting material in the present studies since the plasma level for factor VIII is extremely low and large amounts of starting material were required. Furthermore, bovine factor VIII is more stable than that from other sources such as human plasma. We have also employed glycine ethyl ester (Kisker, 1967) and various serine protease inhibitors to stabilize the factor VIII coagulant activity during the isolation procedure. Nonetheless, continued loss of coagulant activity remained a major problem in the isolation procedure.

Reduction of the factor VIII/vWF preparation yielding an apparent low molecular weight form(s) of the coagulant activity has been reported by several groups (Austen, 1974; Cooper et al., 1975; Brown et al., 1976; Peake & Bloom, 1976; Blombäck et al., 1978). We have utilized this procedure to free the factor VIII coagulant activity from high molecular weight proteins, such as fibrinogen. A number of investigators (Newman et al., 1976; Blombäck et al., 1978; Rock et al., 1978) have suggested that the low molecular weight factor VIII may be the actual form in plasma and the high molecular weight factor VIII may be an artifact of the isolation procedure. Whether the factor VIII described in this report is identical with that present in plasma has not been established.

The purified bovine factor VIII appeared as a triplet upon sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis (Figure 3). Identification of the triplet as factor VIII was supported by the fact that a change in the gel electrophoresis pattern was associated with its activation by thrombin or factor X_a. Furthermore, cleavage of each of the three bands during the activation reaction suggests that all three are closely related proteins and are associated with factor VIII coagulant activity. Also, the sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis pattern for the activated form differs completely from that of the precursor, suggesting that the highly purified form is free of activated factor VIII. The triplet observed on sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis was not due to minor proteolysis during the factor X–Sepharose column chromatography step since this same triplet was observed prior to this affinity step.

Activated factor VIII was stable under the activation conditions employed (low pH and the presence of glycine ethyl ester, calcium chloride, and glycerol). As with the activated form of factor VIII/vWF (Vehar & Davie, 1977), the thrombin-activated form of highly purified factor VIII was inhibited by 0.030 M diisopropyl fluorophosphate.

The inactivation of factor VIII by activated protein C provides additional supporting evidence that the protein triplet observed on gels is indeed factor VIII. Activated protein C is a highly specific protease (Kisiel et al., 1977). It will inactivate both the factor VIII/vWF coagulant activity and the purified factor VIII coagulant activity. With the purified form of factor VIII, inactivation was associated with the cleavage of the highest molecular weight form of the triplet. One interpretation of these data is that the factor VIII clotting activity is due to the 93 000 molecular weight polypeptide chain, and the 88 000 and 85 000 molecular weight polypeptide chains are degradation products resulting from minor proteolysis by activated protein C or some other similar serine protease(s) present in plasma. This is suggested by the fact that no other degradation products were observed. It is also possible, however, that factor VIII is composed of several tightly associated subunits of about 90 000 molecular weight yielding a total molecular weight of 250 000–300 000. This would be consistent with the apparent molecular weight of factor VIII by gel filtration (Figure 2). Activated protein C also rapidly inactivated thrombin-activated factor VIII. This inactivation was associated with a cleavage of the 39 000-dalton fragment. Furthermore, this reaction was faster than that observed with the unactivated form. Whether activated protein C plays a role in the inactivation of factor VIII and factor V under physiological conditions is not known. A combined deficiency of factor V and factor VIII occurs, however, more frequently than can be accounted for by chance. It is possible that this disease is the result of an excessive activation of protein C and/or a decreased amount of an inhibitor for this protein. Theories for the participation of protein C activation in hemophilia can also be postulated but, at present, seem less likely. Further investigation is needed to examine these possibilities.

The coagulant activity of factor VIII/vWF and purified factor VIII was inhibited by antibodies against both the factor

VIII/vWF protein and purified factor VIII. These results suggest that the highly purified factor VIII protein described in this report is responsible for the coagulant properties of the factor VIII/vWF complex. The activity of the factor VIII/vWF protein isolated in this laboratory by the method of Legaz & Davie (1976) has a specific activity of approximately 20 units/mg. On the basis of the specific activity of purified factor VIII (Table I), less than 0.5% of the protein in the factor VIII/vWF preparation is responsible for the coagulant activity. Inhibition of the coagulant activity of purified factor VIII and factor VIII in plasma by increasing concentrations of antibody indicates that the two activities are inhibited to the same extent in the presence of a given amount of antibody. Therefore, the highly purified factor VIII is very similar, and possibly identical, in its antigenic properties to the coagulant protein present in whole plasma.

Bovine factor VIII/vWF will aggregate human platelets (Donati et al., 1973). This reaction is inhibited by antibodies to the factor VIII/vWF protein (Forbes & Prentice, 1973; Meyer et al., 1973; Weiss et al., 1973). The lack of inhibition of platelet aggregation by the antibodies to purified factor VIII provides good evidence for the concept that the coagulant portion of factor VIII does not arise through a processing of the 200 000-dalton subunit of the factor VIII/vWF complex and is a distinct protein. The uniqueness of the two is also demonstrated by the lack of platelet-aggregating activity by the purified factor VIII preparation.

The chemical characterization of the purified factor VIII will require additional studies. In preliminary studies, the adsorption of the highly purified preparations to a concanavalin A–Sepharose column was observed. Furthermore, the coagulant activity was eluted with methyl glucopyranoside. This suggests that the highly purified preparation contains carbohydrate. Also, the exact role of factor VIII in the activation of factor X remains to be clarified. Studies employing the highly purified preparation are currently under investigation in our laboratory to establish, if possible, its role in this reaction.

Acknowledgments

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Maize Histone H1: A Partial Structural Characterization†

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ABSTRACT: The first H1 histone from a plant, *Zea mays*, has been characterized and partially sequenced. The maize H1 molecule shares many sequence features with rabbit and sea urchin H1 and chicken H5. The central hydrophobic region of the protein exhibits sequence microheterogeneity, indicating the presence of multiple H1 proteins in maize. The cause of the genetically controlled electrophoretic variation in the major H1 subfraction of maize has been localized to the carboxy-terminal region of the molecule.

Histone H1 plays an important role in the organization of nucleosomes into higher order structures (Baldwin et al., 1975; Finch & Klug, 1976; Müller et al., 1978). The existence of variability in the H1 fraction of animals and plants suggests that this role may be regulatory in addition to structural. Even the H1 fraction from a single tissue consists of several subfractions which differ slightly in molecular weight and amino acid sequence (Panyim & Chalkley, 1971; Kinkade & Cole, 1966); the number, amount, and species of H1 subfractions vary from tissue to tissue within an organism (Bustin & Cole, 1968) and during development (Ruderman et al., 1974). Intraspecific variation (maize) (Stout & Phillips, 1973) and interspecific variation (Panyim et al., 1971; Spiker, 1975) also exist. These observations suggest that the several species of H1 have specific roles in gene control. Such control could be the result of differences in the primary structures of the several H1 subfractions. This paper examines the extent of H1 variation and presents the first detailed structural analysis of the H1 histones from a plant. The paper compares the subfractions of maize H1 to each other and to animal H1. It also localizes the cause of the genetically controlled electrophoretic variation of maize H1a observed by Stout & Phillips (1973) to the carboxy-terminal region of the H1a molecule.

Materials and Methods

Chromatin and histone were isolated from immature tassels of *Zea mays* as described previously (Stout & Hurley, 1977; Stout & Phillips, 1973). One gram of total histone, preincubated for 1 h at room temperature in 7 M guanidine hydrochloride and 0.1 M Tris base to decrease protein aggregation, was fractionated on a 5 × 270 cm Bio-Gel P100 column in 0.01 N HCl and 1 mM sodium azide (Sommer & Chalkley, 1974).

Amino acid compositions were determined from 24-h 4 N methanesulfonic acid and 6 N HCl hydrolysates (Simpson et al., 1976; Gibson et al., 1971) by using a Durrum D500 amino acid analyzer.

The preparative steps were assessed by electrophoresis in acetic acid, 6.25 M urea, and 15% polyacrylamide slab gels (Hurley, 1977). Size characterizations were carried out on 15 and 20% Laemmli (1970) sodium dodecyl sulfate (NaDodSO$_4$) gels by using calf thymus histones as molecular weight standards.

Cyanogen bromide (CNBr[1]) cleavages were performed by adding the solid CNBr to an equal weight of protein at a

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[1] Abbreviations used: CNBr, cyanogen bromide; NBS, *N*-bromosuccinimide.

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