The Isolation and Characterization of Bovine Factor VIII (Antihemophilic Factor)*

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Gottfried Schmer,† Edward P. Kirby,§ David C. Teller, and Earl W. Davie

From the Department of Biochemistry, University of Washington, School of Medicine, Seattle, Washington 98195

SUMMARY

Bovine Factor VIII (antihemophilic factor) has been purified approximately 10,000-fold with an over-all yield of 10 to 15%. The purification procedure involves BaSO₄, kaolin, and bentonite adsorption to remove contaminants, ethanol, polyethylene glycol, and β-alanine fractionation, calcium citrate-cellulose chromatography, concanavalin A precipitation, and an Agarose gel filtration step. The final product is homogeneous when examined by gel filtration, density gradient centrifugation, and zone electrophoresis. It also shows a single precipitin line when subjected to immunoelectrophoresis employing a specific rabbit antibody against Factor VIII. However, when examined by sedimentation equilibrium, the preparation is physically heterogeneous, apparently due to substantial aggregation of the protein. In these experiments, the smallest species which could be calculated has a molecular weight of approximately 1.1 million. After reduction in 2-mercaptoethanol, the protein shows one band in polyacrylamide gel electrophoresis and zone electrophoresis and a reduction in size. The smallest species of the reduced protein has a molecular weight of about 85,000. The protein contains approximately 11% hexose, 2% sialic acid, 7% hexosamine, and no lipid. Other general properties of this protein including its amino acid composition are also reported.

A careful study of the role of Factor VIII (antihemophilic factor) in blood coagulation is dependent upon a highly purified preparation which has well defined physical-chemical characteristics. Although many different methods of preparation for this protein from various sources have been described during the past 20 years, few have yielded preparations of high quality and in sufficient quantity to permit detailed studies. Most methods of purification have involved ethanol (1), ether (2), or polyethylene glycol (3) fractionation, precipitation with amino acids (4), phosphate (5), citrate (6), and by decreased temperature (7), and removal of fibrinogen contaminants with adsorbents such as bentonite (8). Gel filtration techniques (9) and concanavalin A precipitation (10) have also provided some success in the purification of this protein. Recently, human Factor VIII has been purified approximately 10,000-fold by Hershgold et al. (3) by means of a combination of several of these methods.

In the present communication, we wish to describe a relatively simple method for the purification of milligram quantities of bovine Factor VIII. This procedure leads to a product of high purity and good yield. Various physical-chemical and biological properties of this preparation are also described. Preliminary studies dealing with this work have been published elsewhere (11).

MATERIALS

Heparin sodium salt (Grade I) and ε-amino-n-caproic acid were purchased from Sigma. Benzamidine hydrochloride was obtained from Aldrich. Barium sulfate (x-ray grade) was purchased from Merck (U. S.), and kaolin N. F. was obtained from J. T. Baker Chemical Co. Bentonite was a product from Prolabo, Paris, France. Sodium barbital was purchased from Mallinckrodt Chemical Works. Polyethylene glycol (Carbowax 6000) was obtained from Union Carbide Corp., and imidazole, β-alanine, and calcium citrate (Ca₃(C₆H₅O₂)₄·4 H₂O) were obtained through Matheson Coleman and Bell, Cincinnati, Ohio. Cellulose powder was Whatman CF1 fibered powder, Reeve Angel, Clifton, N. J. The final step of gel filtration was carried out with Agarose A-15m or A-50m supplied by Bio-Rad, Richmond, Calif. Methyl-α-D-glucopyranoside (β grade) was obtained from Calbiochem. Assolethone, purchased from Associated Concentrates Lecithin, Woodside, N. Y., was used as a platelet substitute. Dri Film silicone (SC 87) was a product from General Electric, Waterford, N. Y., and all glassware was coated with this material unless otherwise noted. Human fibrinogen was purchased from Warner-Chilcott, Morris Plains, N. J. Bovine thrombin employed for the fibrinogen assays was...
purchased from the Upjohn Co. Concanavalin A was prepared according to the method of Agrawal and Goldstein (12). Human plasma α1-glycoprotein was kindly provided by Dr. S. I. Halt mori, University of Washington, Seattle, Wash. All other chemicals were commercial preparations of the highest quality available.

**METHODS**

Protein concentration was measured by the ninhydrin reaction following alkaline hydrolysis according to the method of Moore (13) or absorption at 280 nm. The hexose content of Factor VIII was determined by the phenol sulfuric acid method of Dubois et al. (14), using glucose as a reference standard. Hexosamine was determined by the Elson and Morgan reaction (15), employing galactosamine as a standard. For the quantitative determination of sialic acid, the thiobarbituric acid assay of Warren was used (16), employing human α1-glycoprotein as a reference standard. This protein contains approximately 11% sialic acid (17).

Factor VIII was reduced and alkylated by dissolving 5 mg of protein in a total volume of 1.5 ml containing 720 mg of recrystallized urea, 0.08 ml of a 0.1 M Tris-HCl solution, pH 8.6, 0.02 ml of 14 M 2-mercaptoethanol, and 0.6 ml of a 0.1 M EDTA solution, pH 8.6. The above reaction mixture was allowed to stand for 4 hours at room temperature under nitrogen, followed by the addition of 0.2 ml of a 1 x NaOII containing 54 μg of recrystallized monoiodoacetic acid. After 15 min, the solution was dialyzed overnight against 0.01 M sodium phosphate buffer, 0.1 M 2-mercaptoethanol, and 8 M urea at room temperature before being subjected to further analysis. In some experiments, it was passed over a Sephadex G-25 column (2 x 40 cm) previously equilibrated with 5% acetic acid and lyophilized using a Virtis lyophilizer.

**Antibody Preparation**—Rabbits were immunized with either the highly purified Factor VIII or material which had only been purified through the early stages of the procedure (up through the adsorption and elution from tricalcium citrate). Initial immunization was achieved by multisite intramuscular or subcutaneous injection of 1 to 2 mg of protein in the presence of Freund’s complete adjuvant. After 4 to 6 weeks, the rabbits were reinjected at intervals, and then bled. Rejection was intramuscularly in the presence of Freund’s incomplete adjuvant for rabbits immunized against the high purity Factor VIII. For immunization against the lower purity material, rabbits were injected intravenously with albumin-precipitated material.

Blood collected from the rabbits was allowed to clot overnight at room temperature. The serum was treated with BaSO4 (100 mg per ml) and centrifuged. Saturated ammonium sulfate was then added to 88% saturation, the precipitate centrifuged, and redissolved in 0.15 M NaCl to one-half the original serum volume. Precipitation with 33% ammonium sulfate was repeated two times, and the final precipitate dissolved in 0.15 M NaCl to one-half the original volume. This was then dialyzed extensively against 1 mM phosphate buffer, pH 7.4, and a small precipitate removed by centrifugation. Sodium chloride was added to make the solution 0.15 M, and sodium azide added (0.02%) to retard bacterial growth. The antibody was stored either in the cold room or frozen.

**Electrophoresis**—Polyacrylamide disc gel electrophoresis of the reduced Factor VIII was carried out by the general method of Davis (18). In the present experiments, electrophoresis was performed in 8 M urea and 0.025 M Tris-HCl and 0.2 M glycine buffer, pH 8.7. A 3.5% gel was employed and electrophoresis was carried out for 4 hours at 4°. The gels were stained for protein with Amido black and for carbohydrate by the method of Zacharius et al. (19).

Zone electrophoresis was carried out in 0.05 M sodium barbital, pH 8.6, on microscope slides layered with 0.5% Agarose containing 2% polymerized acrylamide as described by Williams and Chase (20). Samples (20 μl containing 10 to 20 μg of protein) were placed in a small well and electrophoresis was carried out at room temperature for 45 min with 150 volts and 5 ma per slide. The slides were stained for protein with Amido black.

Immunoelectrophoresis on 0.5% Agarose containing 2% polyacrylamide on microscope slides (25 X 75 mm) was carried out according to the method of Scheidegger (21). Essentially the same conditions were employed as those for zone electrophoresis. Samples were diluted in 0.05 M sodium barbital buffer, pH 8.6, and run for 45 min. Antibody was added to the center trough and allowed to diffuse for at least 48 hours. The slides were photographed employing indirect lighting.

**Amino Acid Analysis**—Samples for amino acid analysis were prepared by the method of Moore and Stein (22). These samples (2 to 4 mg) were hydrolyzed in 5.8 N constant boiling HCl for 24, 48, 72, and 96 hours. A small black precipitate, presumably due to humin formation, was removed by centrifugation prior to analysis. The protein concentration for each sample was determined by the ninhydrin reaction following alkaline hydrolysis as described earlier since the protein dried in vacuo or by lyophilization formed an insoluble, sticky gum which could not be readily weighed. Amino acid analyses were carried out on 250-μg protein samples using a Spinco model L20 amino acid analyzer according to the method of Spackman et al. (23). The final protein concentration of each sample was calculated by summation of the amino acid composition, and this was in good agreement with that determined on the original sample by the ninhydrin reaction employing leucine as a standard. Samples from three different preparations of Factor VIII were employed and gave essentially identical results. The reported values for serine and threonine are extrapolations to zero time hydrolysis, whereas isoleucine, leucine, and valine values are the average of the 96-hour hydrolysis. Half-cystine and methionine were determined after performic acid oxidation according to Hirs (24). Tryptophan was determined by the method of Benece and Schmid (25).

**Ultracentrifuge Analysis**—Sedimentation equilibrium experiments were performed as described by Harris et al. (26) and Seeley et al. (27). Calculations were made by the methods discussed by Teller et al. (28). All experiments were conducted at 20°. The partial specific volume of the protein moiety was calculated using the amino acid composition according to the method of McMeekin et al. (29). To correct this value for carbohydrate content (see Table IV), a value of 0.62 ml per g was employed (30). Thus, the actual value of ω was calculated as follows: ω = (0.74 ml per g × 0.80) + (0.62 ml per g × 0.20) = 0.72 ml per g. For the experiment in 6 M guanidine HCl, the value was lowered by 0.01 ml per g (31).

Density gradient ultracentrifugation studies of Factor VIII were carried out in a Spinco model L with a SW 39 rotor employing 5-ml cellulose nitrate tubes. The gradient employed was 10 to 35% methyl-a-D-glucopyranoside in 0.2 M perchloric and 0.05 M borate buffer, pH 8.0. Centrifugation time was 5...
hours at 39,000 rpm at room temperature. The Factor VIII solution was collected in 5-drop samples and analyzed for protein by the ninhydrin method and for Factor VIII activity.

Clotting Assays—Platelet-deficient citrated plasma was prepared from normal human blood and from the blood of patients with various coagulative abnormalities by previously described methods (32). In this procedure, plasma is not permitted to come in contact with glass and is frozen at -90° until employed. Pooled platelet-deficient oxalated bovine blood was employed as a reference standard in the Factor VIII assay and was stored at -90°.

Factor VIII activity was measured by the kaolin-activated partial thromboplastin time employing the method of Langdell et al. (33) as modified by Proctor and Rapoport (34). In this assay, 0.1 ml of Factor VIII-deficient plasma (Factor VIII level less than 1%) is previously incubated with 0.1 ml of an appropriate serial dilution of Factor VIII (for the starting plasma 1:10, 1:20, 1:40), 0.1 ml of a 0.1% aseptic solution, and 0.1 ml of a 9% kaolin suspension for 8 min at 37°, followed by the addition of 0.1 ml of 0.025 M CaCl2. The time which is required to form a clot after the addition of Ca2+ is recorded with a stop watch. The tilting method is used with siliconized glass tubes (8-mm internal diameter). A standard reference curve was prepared from the most highly purified product. A straight line was obtained when various concentrations of Factor VIII and their clotting times were plotted on double log paper. The slope of the line made from Factor VIII at different stages of purification was parallel to that obtained with the most highly purified product. For a given set of reagents (i.e. the same deficient plasma, phospholipid, and length of prior incubation), clotting times were readily reproducible. One unit of Factor VIII was arbitrarily chosen as that amount of activity present in 1 ml of fresh bovine plasma. For experiments other than those shown in Table I, the units are only approximate since fresh bovine plasma was not readily available as a reference standard.

Fibrinogen was assayed employing the methods of Jacobsson (35) and Clauss (36), and Factor V activity was measured on artificially depleted Factor V substrate plasma (37). Prothrombin concentrations were determined by the method of Quick et al. (38). Various other coagulation factors (Factors VII, IX, and X) and plasminogen were determined essentially as described by Biggs and Macfarlane (39).

Phospholipid extraction was carried out on 5-ml samples of Factor VIII which were extracted with chloroform-methanol, and the extract dried under nitrogen. The samples were then heated to 210° for 1 hour after the addition of 0.3 ml of 10% perchloric acid. They were then cooled and mixed with 2.5 ml of a solution containing 0.4% ammonium molybdate and 0.2 ml of 8 × 10⁻⁴ M maminonaphthalene sulfonic acid. After heating for an additional hour at 100°, the samples were cooled, brought to 5 ml with H₂O, and the optical density determined at 820 nm. By this method, levels as low as 0.1 mg of phosphate per sample were readily determined. Free and bound fatty acids were determined by gas chromatography according to the method of Ways and Hanahan (39).

Purification of Bovine Factor VIII—Nine liters of bovine blood were collected directly into plastic containers containing 1 liter of 0.10 M oxalate, 0.10 M benzamidine-HCl, and 20,000 units of heparin. Mixing of the blood with the anticoagulant was facilitated by pouring the blood into a second container and into a 20-liter polyethylene vessel. Care was taken so that not more than 1 min elapsed between the death of the animal and the collection of the blood. The blood was transported to the laboratory (usually less than 1 hour) at ambient temperature and centrifuged at 0° for 75 min at 2,500 × g. The plasma was decanted and stirred slowly with BaSO₄ (100 mg per ml) for 15 min at 4°. In this step, the BaSO₄ adsorbs prothrombin and Factors VII, IX, and X. The suspension was centrifuged at 0° for 15 min at 2,500 × g, and the supernatant was treated with kaolin (100 mg per ml) for 15 min to remove the major portion of the contact factors (Factors XI and XII). The plasma was then centrifuged as above and stored at -80° in 2-liter plastic containers. Under these conditions, the BaSO₄-kaolin-adsorbed plasma can be stored for months without losing Factor VIII activity.

For further purification, 4 liters of deep-frozen BaSO₄-kaolin-adsorbed plasma were thawed overnight at room temperature. After about 12 hours, a small amount of cryoprecipitate was apparent and some ice still remained. The melting plasma was stirred for an additional 30 min and adjusted to a pH of 6.3 with a KH₂PO₄ solution saturated at room temperature. This usually amounts to about 100 ml of phosphate solution per preparation. Ethanol (100% precooled to -90°) was slowly added with stirring to give a final concentration of 8%, and the plasma which still contained some ice was poured into plastic centrifugation cups and placed in the centrifuge at -3° for 10 min prior to centrifugation. The solution was centrifuged at 2,500 × g for 10 min at -3°, and the precipitate containing Factor VIII was suspended in 800 ml of 0.02 M imidazole-HCl buffer, pH 6.5, at 0° for 5 min. The washed precipitate was centrifuged at 2,500 × g for 10 min at 0° and dissolved in 200 ml of 0.25 M NaCl solution containing 0.02 M imidazole-HCl, pH 6.5, and 0.02 M ε-amino caproic acid by gently stirring at room temperature for about 30 min. Bentonite solution (40 ml) (100 mg per ml in 0.15 M NaCl) was then added slowly with stirring. After an additional 10 min, the bentonite was removed by centrifugation at 10,000 × g at 22° for 10 min. Twenty per cent polyethylene glycol in 0.25 M NaCl-0.02 M imidazole, pH 6.5, was added slowly to the supernatant to make the bentonite-adsorbed supernatant 4%, with respect to polyethylene glycol, and the solution was left for 10 min at room temperature after the addition of the last drop of polyethylene glycol. A precipitate was removed by centrifugation at 4,000 × g for 10 min, and this contained most of the Factor VIII activity. Centrifugation at this lower speed provided a pellet which was more easily dissolved than if spun at high speed.

The precipitate was redissolved in 50 ml (one-eighth of original volume) of 0.05 M citrate, pH 6.8, containing 0.02 M ε-amino caproic acid by warming in a water bath at 37° under slight agitation. The final volume of the solution was measured and an equal volume of 3 M β-alanine solution in 0.05 M citrate, pH 6.8, was added dropwise with stirring. With the last few milliliters of β-alanine solution, a precipitate begins to form. The solution again was left for 10 min at room temperature and centrifuged at 10,000 × g for 10 min at 22°. The resulting precipitate containing the Factor VIII activity was redissolved in 40 ml (one-hundredth of original volume) of 0.05 M citrate and 0.02 M ε-amino caproic acid at 37° and further purified by chromatography at room temperature on a calcium citrate-cellulose column. In this procedure, 20 g of calcium citrate were
mixed with 20 g of cellulose in 0.05 M citrate, pH 6.8, and poured into a siliconized column (2.5 × 30 cm). After the column had settled, the solution from the β-alanine step was applied to the column. The column was extensively washed with the same citrate buffer until the extinction of the effluent was less than 0.03. This usually required about 1 liter of citrate buffer.

After the citrate wash, the column was placed in the cold room at 4° and the Factor VIII activity was eluted slowly at 4° by a solution containing 0.1 M ethylenedinitrilo-tetraacetic acid tetra-citric acid salt, 0.2 M Tris HCl, and 0.02 M amino caproic acid adjusted to a final pH of 8.6. The elution rate did not exceed 30 ml per hour.

The eluate containing the Factor VIII activity was mixed with an equal volume of 20% polyethylene glycol in 0.25 M NaCl and 0.02 M imidazole buffer, pH 6.5, at 4°. After standing in an ice water bath for 30 min, a small precipitate containing all the Factor VIII activity was centrifuged at 0° at 37,000 g for 10 min.

For final purification, the precipitate was dissolved in approximately 10 ml of 0.2 M KCl-0.02 M Tris HCl, pH 7.4, at 22°. The absorbance at 280 nm was determined on a 0.1-ml aliquot which had been diluted with 0.8 ml of the same buffer. The resulting optical density was multiplied by one-half the volume of the Factor VIII solution. This is equivalent to the volume of concanavalin A solution (9.0 O.D. units per ml; i.e. 7.9 mg per ml in 0.15 M NaCl) to be added. The concanavalin A was added with stirring at room temperature and the solution was left to stand for 15 min. The flocculent precipitate consisting of a concanavalin A-glycoprotein complex was centrifuged at 10,000 g for 10 min. This prepara
tion was then stored in the cold room for several months, or may be frozen without loss of activity.

During the early studies, the purification was carried out in 3 days by stopping after the kaolin step on the 1st day and the calcium citrate step on the 2nd day. These preparations of Factor VIII were prepared from 4-liter batches of plasma. More recently, 16 liters of plasma have been employed routinely throughout the whole procedure and the final yield has been 25 to 30 mg of Factor VIII. This high yield (about 15 to 20%) is obtained by continuing the purification procedure from the cryoprecipitate up to the dissolving of the concanavalin A precipitate in 1 day. This requires about 18 hours of work, starting early in the morning. The higher yield is presumably due to less proteolysis at the early stages of purification and better recoveries from the precipitates.

After each precipitation step, the Factor VIII precipitate was slurried in a small volume of buffer and extracted with successive washes of buffer until it completely dissolved. It was important to dissolve all precipitates immediately, since on standing they became more difficult to solubilize and recoveries were poor.

The Factor VIII purification procedure has been employed in our laboratory at least 50 times during the past 2 years with only a few failures. Once completely purified, the Factor VIII is stable in solution in the cold room for several months, or may be frozen without loss of activity.

RESULTS

Preparation of Bovine Factor VIII—The steps in the purification procedure for bovine Factor VIII and the yield from a typical preparation are shown in Table I. The procedure involves BaS0₄, kaolin, and bentonite adsorption for the removal of prothrombin, Factors VII, IX, and X, and kaolin and bentonite adsorption for the removal of fibrinogen and the contact factors (Factors XI and XII).

The removal of fibrinogen from Factor VIII preparations has always been a major problem in the purification of this protein. In the present procedure, fibrinogen is removed at three different steps (see Table I). The calcium citrate-cellulose column removes the last portion of the fibrinogen and is one of the most effective purification steps in the entire procedure. The use of calcium citrate-cellulose was originally described by Blomback et al. (40) as a batch procedure for the purification of human Factor VIII, but in a later publication, these authors reported less success with this method (41). The poor yield which they obtained may have been due in part to a substantial decrease in pH

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein concentration</th>
<th>Volume</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total units</th>
<th>Percentage of yield</th>
<th>Purification</th>
<th>Percentage of fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>70 mg/ml</td>
<td>4,400 ml</td>
<td>308,000 mg</td>
<td>0.014 units/mg</td>
<td>4,400 units</td>
<td>100 %</td>
<td>1</td>
<td>100 %</td>
</tr>
<tr>
<td>BaS0₄-kaolin</td>
<td>56 mg/ml</td>
<td>4,000 ml</td>
<td>200,000 mg</td>
<td>0.0176 units/mg</td>
<td>4,000 units</td>
<td>90 %</td>
<td>1.2</td>
<td>40 %</td>
</tr>
<tr>
<td>Ethanol</td>
<td>55 mg/ml</td>
<td>2,000 ml</td>
<td>11,000 mg</td>
<td>0.28 units/mg</td>
<td>3,080 units</td>
<td>70 %</td>
<td>20</td>
<td>40 %</td>
</tr>
<tr>
<td>Bentonite</td>
<td>15.7 mg/ml</td>
<td>240 ml</td>
<td>2,770 mg</td>
<td>0.7 units/mg</td>
<td>2,640 units</td>
<td>60 %</td>
<td>50</td>
<td>10 %</td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>15.7 mg/ml</td>
<td>100 ml</td>
<td>1,570 mg</td>
<td>1.4 units/mg</td>
<td>2,200 units</td>
<td>50 %</td>
<td>100</td>
<td>10 %</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>15.7 mg/ml</td>
<td>50 ml</td>
<td>785 mg</td>
<td>2.8 units/mg</td>
<td>2,200 units</td>
<td>50 %</td>
<td>200</td>
<td>10 %</td>
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<tr>
<td>Calcium citrate-cellulose</td>
<td>15.6 mg/ml</td>
<td>30 ml</td>
<td>47 mg</td>
<td>26 units/mg</td>
<td>1,930 units</td>
<td>20 %</td>
<td>2,000</td>
<td>0 %</td>
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<tr>
<td>Concanavalin A</td>
<td>13 mg/ml</td>
<td>2 ml</td>
<td>26 mg</td>
<td>42 units/mg</td>
<td>1,100 units</td>
<td>25 %</td>
<td>3,000</td>
<td>0 %</td>
</tr>
<tr>
<td>Agarose</td>
<td>0.2 mg/ml</td>
<td>15 ml</td>
<td>3.1 mg</td>
<td>140 units/mg</td>
<td>440 units</td>
<td>10 %</td>
<td>10,000</td>
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Fig. 1. Gel filtration of partially purified Factor VIII on Agarose A-50m (top panel) and Agarose A-15m (bottom panel). A 1-ml solution of the Factor VIII preparation (10 mg per ml in 0.25 M NaCl-0.02 M imidazole buffer, pH 6.5, and 10% methyl-\(\alpha\)-d-glucopyranoside) from the concanavalin A step was applied to each column (1.5 X 90 cm) as described under "Methods." \(\bullet\)--\(\bullet\), Factor VIII activity; \(\Delta\)--\(\Delta\), protein concentration as measured by the ninhydrin method.

Fig. 2. Gel filtration of the purified Factor VIII on Agarose A-50m (top panel) and agarose A-15m (bottom panel). A 1-ml solution of the Factor VIII preparation (6 mg per ml in 0.25 M NaCl-0.02 M imidazole buffer, pH 6.5, and 10% methyl-\(\alpha\)-d-glucopyranoside) from each of the gel filtration steps shown in Fig. 1 was applied to a second corresponding Agarose A-50m or Agarose A-15m column (1.5 X 90 cm). In these experiments, the carbohydrate analysis was carried out as described under "Methods" after extensive dialysis of each sample against 0.25 M NaCl and 0.025 mM imidazole buffer, pH 6.5, to remove free methyl-\(\alpha\)-d-glucopyranoside. \(\bullet\)--\(\bullet\), Factor VIII activity; \(\Delta\)--\(\Delta\), protein concentration; \(\square\)--\(\square\), carbohydrate concentration.

Fig. 3. Density gradient centrifugation of Factor VIII. Factor VIII (0.7 mg in 0.2 ml of 0.2 M sodium perchlorate and 0.05 M borate buffer, pH 8.0) was layered on a gradient of 10 to 35% methyl-\(\alpha\)-d-glucopyranoside and centrifuged as described under "Methods." \(\Delta\)--\(\Delta\), protein; \(\bullet\)--\(\bullet\), Factor VIII activity.

which occurs during protein elution from calcium citrate when it is carried out at low buffer concentrations. This problem was avoided by employing 0.20 M Tris buffer, pH 8.6, in the present experiments.

The use of concanavalin A in the purification of human Factor VIII was first described by Kass et al. (10) but abandoned in a purification procedure which was published later (42). In preliminary experiments, concanavalin A was covalently linked to Sepharose 2B by the cyanogen bromide method of Porath et al. (43). With this procedure, optimal conditions for binding and elution of bovine Factor VIII were established by column chromatography experiments. Subsequently, free concanavalin A was used in a batch process since this gave better recoveries. The concanavalin A step also removed the remainder of the Factor V which was present in trace amounts after the calcium citrate-cellulose step.

The contaminants present after the concanavalin A precipitation step are removed by gel filtration on Agarose. A typical elution profile for gel filtration on Agarose is shown in Fig. 1. The top portion of the figure shows the results with Agarose A-50 m, and the bottom portion of the figure shows the results with Agarose A-15m. In these experiments, Factor VIII appears in the first protein peak from both columns, followed by a second contaminating peak which includes concanavalin A. Gel filtration of the first protein peak from Agarose A-15m or A-50m shown in Fig. 1 gives a single symmetrical peak (Fig. 2). In these experiments, a close correlation between protein, Factor VIII activity, and carbohydrate was observed. These experiments suggest that bovine Factor VIII is a high molecular weight glycoprotein, and the final purification step yields a preparation which is homogeneous by the criteria of gel filtration.

This preparation is also free of all other known clotting factors. This was shown by testing 0.1 ml of an undiluted Factor VIII solution (1 mg of protein per ml, approximately 140 units of Factor VIII per ml) for fibrinogen, prothrombin, and Factors V, VII, IX, X, XI, and XIII. It was also free of detectable plasminogen.
One of the major reasons for the loss of Factor VIII activity in plasma is its destruction by proteolytic enzymes (44). The present procedure employs blood collected in oxalate, heparin, and benzamidine to limit proteolysis. The addition of heparin substantially improves the yield of Factor VIII apparently by further inhibiting the coagulation process. Thrombin and other serine proteases, such as plasmin, are also inhibited by benzamidine (45), and the addition of this inhibitor increases the over-all yield to 10 to 15% for Factor VIII. In the absence of benzamidine and heparin, the final yield is less than 5%.

Another major loss of Factor VIII occurs by adsorption of the protein to various surfaces, particularly at the higher stages of purification. This was especially evident during the calcium citrate-cellulose column chromatography and the final gel filtration step on Agarose. In each of these steps, there was a 40 to 60% loss in Factor VIII activity. Losses by glass adsorption, however, can be avoided to a major extent by employing silicone-coated glassware during purification and storage.

Density Gradient Centrifugation of Bovine Factor VIII—Density gradient centrifugation was carried out on Factor VIII to provide further evidence for the purity of this clotting factor preparation. In these studies, the protein was centrifuged through a methyl-α-D-glucopyranoside gradient of 10 to 35% (w/v) in the presence of 0.05 M borate buffer, pH 8.0, and 0.2 M sodium percolate to minimize aggregation (46). Centrifugation was carried out at room temperature since aggregation and precipitation of Factor VIII occur at 4°C even in the presence of a strong chaotropic agent like perchlorate. As shown in Fig. 3, a close parallelism is observed between activity and protein concentration throughout the gradient.

Zone Electrophoresis—Factor VIII was then subjected to electrophoresis at pH 8.6, employing microscope slides layered with Agarose-nopolymerized acrylamide (Fig. 4). After electrophoresis for 45 min, a single spot was observed for Factor VIII (Sample 1). Upon reduction with 2-mercaptoethanol in 8 M urea (Sample 2) or reduction and subsequent alkylation with iodoacetate (Sample 3), single spots were also obtained. The distance of migration for the reduced and alkylated fractions is increased relative to the native molecule. Also, the addition of carboxymethyl groups by alkylation slightly increases the mobility of this fraction relative to the reduced form.

Immunoelectrophoresis—As another criterion for purity, Factor VIII was subjected to electrophoresis on Agarose-acrylamide slided, followed by immunodiffusion against rabbit antibody prepared from a highly purified Factor VIII preparation. In these experiments, Factor VIII migrates toward the anode during electrophoresis and forms a single sharp precipitin line after the addition of antibody to the center trough (Fig. 5). Three different Factor VIII preparations are shown and each shows only a single precipitin line.

Immunoelectrophoresis experiments were also carried out with an antibody prepared from a partially purified Factor VIII preparation. This sample contained about 10 to 20% Factor VIII and was obtained from the calcium citrate-cellulose column step. In these experiments shown in Fig. 6, the top well contained a purified Factor VIII preparation, and the bottom well contained Factor VIII purified only through the calcium citrate-cellulose column step. It is clear from these studies that the crude Factor VIII preparation shows many precipitin lines, and these are reduced to only one after the final purification step.

The antibody employed in these experiments readily neutralized Factor VIII activity as measured in the regular Factor VIII assay (Table II). In these experiments, the antibody and Factor VIII were previously incubated together and aliquots were assayed in Factor VIII-deficient plasma. The neutralization of Factor VIII activity at the higher antibody concentration was greater than 90%. Similar results were obtained with the antibody prepared from the partially purified Factor VIII preparation.

These experiments support the conclusion that the single protein precipitin line observed in the immunoelectrophoresis experiments (Figs. 3 and 6) is due to the presence of Factor VIII. Furthermore, they provide strong evidence for the purity of the bovine Factor VIII preparation.

Polyacrylamide Gel Electrophoresis of Bovine Factor VIII—In preliminary studies, it was observed that bovine Factor VIII does not enter a 3.25% polyacrylamide gel even in the presence of 8 M urea. After reduction of Factor VIII with 2-mercaptoethanol, however, a single protein and a single carbohydrate band are obtained following polyacrylamide gel electrophoresis (Fig. 7). In these experiments, the protein was detected with Amido black and the carbohydrate with the periodic acid-Schiff reagent. These results suggest that Factor VIII is made of subunits held together by disulfide bonds, and these subunits are very similar in size or are perhaps identical.

Sedimentation Equilibrium Studies of Bovine Factor VIII—Sedimentation equilibrium results demonstrated that Factor VIII was physically heterogeneous both in the native and reduced states (Table III). For the native protein, the two most concentrated samples (0.50 and 0.75 mg per ml) appeared to be in chemical equilibrium, but the most dilute sample (0.25 mg per ml) displayed much higher molecular weights. This result could arise from either computation errors or a lack of chemical equilibrium among all species (26). This data caused the rather large standard deviations of $M_o$ and $M_s$ shown in Table III. The smallest species which could be calculated under these conditions has a molecular weight in excess of $10^6$ g per mole. In the presence of 8 M urea, the molecular weight was also greater than $10^6$ g per mole. In addition, a single sedimentation velocity experiment using absorption optics displayed physical heterogeneity as seen by some spreading of the boundary.

In an attempt to determine subunit molecular weights, Factor VIII was centrifuged to equilibrium in both 8 M urea and 6 M guanidine-HCl containing 2-mercaptoethanol. The experiment in urea showed poor precision and the molecular weights ob-
platted experiments, Factor VIII antibody was prepared against a partially purified Factor VIII preparation obtained from the calcium citrate-cellulose column. Electrophoresis was carried out as described in the legend to Fig. 5. Following electrophoresis, 50 μl of rabbit Factor VIII antibody solution (1.45 mg per ml) were added to the center trough and photographs were taken after 72 hours at room temperature. The anode is at the left of the photograph. The well on the top of the slide contained the highly purified Factor VIII, and the well on the bottom of the slide contained the partially purified Factor VIII.

Fig. 7 (right). Polyacrylamide gel electrophoresis of reduced bovine Factor VIII. Protein (25 μg) was applied to each gel as described under "Methods." The gel on the left was stained for protein with Amido black and the gel on the right was stained for carbohydrate with the periodic acid-Schiff reagent.

Lipid Content of Bovine Factor VIII—Hershgold et al. (3) reported a lipid content of human Factor VIII of 11%. Also,
TABLE II
Neutralization of Factor VIII activity by Factor VIII antibody

<table>
<thead>
<tr>
<th>Additions</th>
<th>Clotting time</th>
<th>Percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>74.7, 75.6</td>
<td>0</td>
</tr>
<tr>
<td>Antibody (0.37 µg)</td>
<td>77.8, 78.4</td>
<td>20</td>
</tr>
<tr>
<td>Antibody (0.73 µg)</td>
<td>83.2, 83.9</td>
<td>42</td>
</tr>
<tr>
<td>Antibody (1.45 µg)</td>
<td>93.0, 92.8</td>
<td>66</td>
</tr>
<tr>
<td>Antibody (2.9 µg)</td>
<td>99.6, 98.6</td>
<td>74</td>
</tr>
<tr>
<td>Antibody (14.5 µg)</td>
<td>122.8</td>
<td>93</td>
</tr>
</tbody>
</table>

* M₁ refers to the smallest species which could be detected calculated by the methods described by Teller et al. (28). The uncertainties are weighted root mean square values in this column, but standard deviations for all other values.

Buffer: 0.01 M Tris-HCl-0.2 M KCl, pH 7.5.
Buffer: 0.04 M Tris-HCl-0.02 M guanidine-HCl-0.2 M 2-mercaptoethanol, pH 7.5.

TABLE III
Molecular weight of bovine Factor VIII by sedimentation equilibrium

<table>
<thead>
<tr>
<th>Sample</th>
<th>rpm</th>
<th>η</th>
<th>Molecular weights (× 10⁵ g/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>M₃</td>
</tr>
<tr>
<td>Nativea</td>
<td>4,027</td>
<td>0.728</td>
<td>2.180 ± 170</td>
</tr>
<tr>
<td>Denatured and reduced in 2-mercaptoethanolb</td>
<td>13,922</td>
<td>0.718</td>
<td>143 ± 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Additions</th>
<th>Clotting time</th>
<th>Percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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<td>0</td>
</tr>
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<td>77.8, 78.4</td>
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</tr>
<tr>
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<td>83.2, 83.9</td>
<td>42</td>
</tr>
<tr>
<td>Antibody (1.45 µg)</td>
<td>93.0, 92.8</td>
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* M₁ refers to the smallest species which could be detected calculated by the methods described by Teller et al. (28). The uncertainties are weighted root mean square values in this column, but standard deviations for all other values.

Buffer: 0.01 M Tris-HCl-0.2 M KCl, pH 7.5.
Buffer: 0.04 M Tris-HCl-0.02 M guanidine-HCl-0.2 M 2-mercaptoethanol, pH 7.5.

TABLE IV
Carbohydrate content of bovine Factor VIII

Carbohydrate and amino acid analyses were carried out on three different preparations of bovine Factor VIII as described under “Methods.”

<table>
<thead>
<tr>
<th>Component</th>
<th>µg/100 µg protein</th>
<th>Per cent</th>
<th>Approximate number of sugar residues/100,000 g glycoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acidsa</td>
<td>100</td>
<td>80.4</td>
<td>61.6</td>
</tr>
<tr>
<td>Hexoseb</td>
<td>13.8</td>
<td>11.1</td>
<td>5.7</td>
</tr>
<tr>
<td>Sialic acidc</td>
<td>2.1</td>
<td>1.7</td>
<td>32.2</td>
</tr>
</tbody>
</table>

a Calculated from data from the amino acid analyzer.
b Calculated as glucose.
c Calculated as N-acetylneuraminic acid.
d Calculated as N-acetylgalactosamine.

denote X-acetylneuraminic acid.

e Calculated from data from the amino acid analyzer.

The amino acid composition for bovine Factor VIII is shown in Table V. The levels of tryptophan and tyrosine in this protein are lower than that found in many other plasma proteins. These low levels account for the decreased absorption at 278 nm for Factor VIII as compared to bovine fibrinogen. This was evident from the 278:260 nm absorption ratios for the two proteins in dilute phosphate buffer at neutral pH. This value is 1.7 for fibrinogen and 1.3 for Factor VIII.

Stability of Purified Bovine Factor VIII—A rather surprising property of the highly purified Factor VIII preparation is its stability. Preparations stored in 0.25 M NaCl-0.02 M imidazole buffer, pH 6.5, and 10% methyl-α-D-glucopyranoside or even in 0.05 M borate, pH 8.0, 0.2 M perchlorate and 25% methyl-α-D-glucopyranoside at 4° do not show any appreciable loss of activity after 2 months. One preparation has been stored at 4° in silicone tubes for 6 months without showing any detectable loss of activity. This stability is observed even in the absence of a protease inhibitor and does not require the addition of methyl-α-D-glucopyranoside.

The highly purified Factor VIII is also rather stable in the general physiological pH range. For instance, incubation at room temperature for 15 min in the pH range of 6 to 8.5 resulted in no loss of activity. However, at further pH extremes (pH 5.0 or 9.0), a rapid decrease of Factor VIII activity occurred.
Tryptophan
Phenylalanine
Tyrosine
Leucine
Threonine
Arginine
Methionine
Valine
Aspartic acid
Lysine
Isoleucine
Half-cystine
Glutamic acid
Histidine
Glycine
Residues/100 g protein

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>µg/100 µg protein</th>
<th>Residues/100 g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>6.89</td>
<td>53.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.06</td>
<td>22.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.10</td>
<td>45.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.80</td>
<td>85.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.44</td>
<td>43.6</td>
</tr>
<tr>
<td>Serine</td>
<td>4.49</td>
<td>61.5</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10.90</td>
<td>84.4</td>
</tr>
<tr>
<td>Proline</td>
<td>4.86</td>
<td>50.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.91</td>
<td>68.5</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.46</td>
<td>49.0</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>5.95</td>
<td>57.6</td>
</tr>
<tr>
<td>Valine</td>
<td>9.84</td>
<td>99.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.30</td>
<td>11.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.12</td>
<td>36.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.20</td>
<td>90.3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.01</td>
<td>16.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.97</td>
<td>26.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3.26</td>
<td>17.5</td>
</tr>
</tbody>
</table>

As mentioned earlier, Factor VIII is readily adsorbed to surfaces such as glass. When bound to glass, it also retains its biological activity as shown in Table VI. In these experiments, an aliquot from a Factor VIII stock solution was transferred into a Pyrex glass tube, incubated for 5 min, and then transferred to a second, third, and fourth Pyrex tube after the same incubation period. After thorough rinsing of each of the Pyrex tubes, residual Factor VIII activity bound to the test tubes was determined.

As seen in Table VI, about half of the original activity is adsorbed to the first two glass tubes and little is adsorbed to the third and fourth tubes. When solutions containing higher levels of Factor VIII are incubated with Pyrex glass tubes under similar conditions, even larger amounts of Factor VIII are adsorbed and the partial thromboplastin time of added hemophilic plasma in these tubes is readily corrected to normal levels. Thus far, we have been unsuccessful in removing Factor VIII in a native form from the glass surface to utilize this property as a purification step.

**DISCUSSION**

The present data indicate that bovine Factor VIII is a glycoprotein present in plasma at a concentration of about 7 µg per ml. The purification procedure described results in a preparation purified approximately 10,000-fold. Establishing the purity of a large molecule such as Factor VIII is difficult, especially when the protein tends to absorb nonspecifically to surfaces such as glass, cellulose, or Sephadex derivatives. Nevertheless, we were able to demonstrate that the final product is homogeneous when examined by gel filtration, density gradient centrifugation, zone electrophoresis, and immunoelectrophoresis. When reduced with 2-mercaptoethanol, it shows a single protein and carbohydrate band on polyacrylamide gel electrophoresis. The preparation, however, is physically heterogeneous when subjected to sedimentation equilibrium studies. In view of the other evidence for purity, it appears probable that the heterogeneity observed in the sedimentation equilibrium experiments is due to aggregation of a chemically pure species.

The presence of hexose, hexosamine, and sialic acid in bovine Factor VIII is typical of plasma proteins. Preliminary results employing gas chromatography have shown that the only two hexoses present were mannose and galactose, and these were found in a ratio of 1:3. Acetylgalactosamine was tentatively identified as the galactosamine. Further studies will be required, however, to confirm these results.

In contrast to human Factor VIII (47), the bovine preparation contains no lipid, as shown by the absence of fatty acids and phospholipid. Factor VIII, however, requires phospholipid during its interaction with factor IXa (48). Thus, it is not surprising to find lipid in human preparations that are made by procedures that do not include ethanol fractionation. The destruction of human Factor VIII by phospholipase C (47), however, suggests that the lipid may have an additional function in the human preparation.

The present studies also show that bovine Factor VIII is a large molecule with a molecular weight greater than one million. The large size of human Factor VIII has been observed by others by gel filtration studies in which the molecular weight was estimated to be larger than two million (10, 49). In the present experiments, there were no indications of Factor VIII activity associated with molecules of smaller molecular weight. Indeed,
attempts to dissociate the molecule into subunits by urea, guanidine-HCl, or chaotropic agents have been unsuccessful. In the presence of a reducing agent, such as 2-mercaptoethanol, the molecule is broken into subunits, the smallest species having a molecular weight of about 85,000. This treatment which breaks disulfide bonds completely destroys the biological activity of Factor VIII.

Other workers (50-53) have reported that Factor VIII activity can be markedly increased by incubation with trace amounts of thrombin. This increase in activity induced by thrombin makes it difficult to compare the present preparation of Factor VIII with those of other investigators whose high specific activity may be due in part to thrombin modification of Factor VIII.

The native bovine Factor VIII prepared by this procedure contains about 140 units of Factor VIII per mg of protein. Incubation with traces of highly purified thrombin causes a 50-fold increase in apparent Factor VIII activity with a final specific activity approaching 7,000 units per mg. This increase in activity is no larger or greater than that seen by other workers and suggests that the activity of our native preparation has not been made spuriously high by inadvertent exposure to traces of thrombin during purification.

Bovine Factor VIII also reacts with Factor IX, in the presence of phospholipid and calcium to form a complex capable of activating Factor X (54). A manuscript describing the details of these experiments is in preparation.

Acknowledgments—We wish to express our sincere thanks to Drs. Arthur Thompson, Robert Meyer, and Richard Counts for valuable discussions and assistance, and to Mark Legaz for excellent technical assistance at various stages of this project. We are also indebted to Dr. W. F. Kretsingcr and the Coagulation Laboratory for kindly providing the bovine plasma employed in these studies.

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Gottfried Schmer, Edward P. Kirby, David C. Teller and Earl W. Davie


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