Isolation and Characterization of Human Factor VIII (Antihemophilic Factor)*

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SUMMARY

Factor VIII (antihemophilic factor) has been purified approximately 500-fold from the cryoprecipitate fraction of human plasma. The isolation procedure involves adsorption of contaminants with Al(OH)₃ column chromatography on tricalcium citrate-cellulose, precipitation with concanavalin A, and an agarose gel filtration step. The final product is homogeneous when examined by zone electrophoresis, sedimentation equilibrium, and immunoelectrophoresis. The molecular weight determined by sedimentation equilibrium is 1.12 million ± 98,000. After reduction of the protein by 2-mercaptoethanol or dithiothreitol, subunits are formed which migrate as one band in polyacrylamide gel electrophoresis and zone electrophoresis. The subunits are heterogeneous, however, in the ultracentrifuge, apparently due to substantial aggregation. The smallest species which could be detected has a molecular weight of 105,000 ± 5,000. The molecular weight of the subunit determined by sodium dodecyl sulfate (SDS) gel electrophoresis was 240,000. The latter value may be high, however, due to the fact that human Factor VIII contains approximately 6% carbohydrate (hexose, hexosamine, and neuraminic acid) and the molecular weights of glycoproteins determined by SDS gel electrophoresis tend to be high. Antibodies prepared in rabbits against human Factor VIII inhibit both human and bovine Factor VIII activity. Antibodies to the highly purified human Factor VIII also form a precipitin line in immunoelectrophoresis experiments with the cryoprecipitate fraction prepared from hemophilic plasma, indicating that an abnormal Factor VIII molecule is present in the plasma of individuals with classic hemophilia. Other general properties of human Factor VIII, including its amino acid composition, thrombin modification, and turnover in hemophilic dogs, are also reported.

(4). It is inactive in individuals with classic hemophilia, a disease which is characterized by a sex-linked coagulation disorder. Factor VIII isolated from bovine plasma is a large glycoprotein with a molecular weight of approximately 1.2 million (5). It is composed of a number of small subunits which are similar or identical, and these are held together by disulfide bonds. Factor VIII has also been highly purified from human plasma. Hershgold et al. (6) purified human Factor VIII approximately 10,000-fold by a procedure which included polyethylene glycol fractionation and a gel filtration step. More recently, Marchesi et al. (7) have reported the isolation of human Factor VIII by a method which includes a limited digestion step with chymotrypsin for the removal of fibrinogen. The purification of bovine Factor VIII, as developed in our laboratory, involved a key step employing calcium citrate-cellulose column chromatography (5). This procedure was very effective in separating bovine Factor VIII from fibrinogen. In the present manuscript, we report the isolation and characterization of human Factor VIII from Blood Bank cryoprecipitate by a procedure which also includes chromatography on a calcium citrate-cellulose column. With this procedure, a relatively stable product of high purity can be made in milligram quantities. This preparation is suitable for various physicochemical and biological studies, many of which are also reported in this paper.

EXPERIMENTAL PROCEDURE

Materials

Aluminum hydroxide gel (Amphojel without flavor) was purchased from Wyeth Laboratories and rabbit brain cephalin, ε-amino-n-caproic acid, Tris (Trizma base), N-acetylneuraminic acid, and p-dimethylaminobenzaldehyde were purchased from Sigma. Barium sulfate (x-ray grade) was purchased from Merck (U.S.), and kaolin (acid wash) was obtained from Fischer Scientific Co. Sodium barbital and 3-sulfosalicylic acid were purchased from Mallinckrodt Chemical Works. Sodium azide N,N',N'-methylenebisacrylamide, polyacrylamide, and N,N',N'-tetramethylethylenediamine (TEMED) were products of Eastman Organic Chemicals. Polyeethylene glycol (Carbowax 6000) was obtained from Union Carbide Corp., and imidazole, (ethylenedinitrilo)-tetraacetic acid, tetrasodium salt (EDTA), ammonium persulfate, and calcium citrate [Ca₂(C₆H₇O₇)₂·2 H₂O] were obtained through Matheson, Coleman and Bell. Whatman CF11 fibrous powder was obtained from Reeve Angel. The final gel filtration step was carried out with agarose...
A-15m supplied by Bio Rad. Agarose, purchased from Marine Colloids, Inc., Springfield, N. J., was employed for zone electrophoresis and immunoelectrophoresis. Methyl-α-D-glucopyranoside (B grade) and dithiothreitol were obtained from Calbiochem. Centrolex-P, purchased from Central Soya, Chicago, Ill., was used as a platelet substitute. Dri Film silicone (SU 87) was a product from General Electric Co., and all glassware was coated with this material.

The 5,5'-dithiobis(2-nitrobenzoic acid) was obtained from Aldrich Chemical Co., Inc. Bovine thrombin was purchased from Parke Davis and Co. and further purified by the method of Thompson and Davie (8). Concanavalin A was prepared according to the method of Agrawal and Goldstein (9). Some of these preparations apparently contained protease activity which readily destroyed Factor VIII activity, and these preparations were heated at 56° for 30 min to overcome this problem. Bovine Factor VIII was prepared by the method of Schmer et al. (5).

All other chemicals were commercial preparations of the highest quality available.

Methods

Protein concentration was measured by the ninhydrin reaction after alkaline hydrolysis according to the method of Moore (10), absorption at 280 nm, or by the method of Lowry et al. (11). The hexose content of Factor VIII was determined by the phenolsulfuric acid method of Dubois et al. (12), employing a 1:1 mixture of glucose and galactose as a reference standard. Hexosamine was determined by the Elson and Morgan reaction as described by Gardea11 (13), with galactosamine as a standard. For the quantitative determination of sialic acid, the thiobarbituric acid assay of Warren (14) was used, with N-acetylneuraminic acid as a reference standard.

Factor VIII was reduced and alkylated by dissolving 5 mg of protein in a total volume of 1 ml containing 720 mg of recrystallized urea, 0.1 ml of a 0.1 M EDTA solution, pH 8.6, 0.015 ml of 14 M 2-mercaptoethanol, and 0.6 ml of a 0.1 M Tris-HCl 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 1 N NaOH containing 56 mg of recrystallized monochloroacetic acid. After 15 min, the solution was dialyzed at room temperature overnight against a solution containing 0.2 M 2-mercaptoethanol, 8.0 M urea, and 0.5 M sodium barbital buffer, pH 8.6. This preparation was then employed directly for zone electrophoresis experiments. For polyacrylamide gel electrophoresis, the protein was reduced with 0.005 M dithiothreitol or 0.2 M 2-mercaptoethanol in 0.1 M phosphate buffer, pH 7.0, containing 9% SDS; 0.002 M EDTA, and 4.0 M urea.

Antibody Preparation—Rabbits were immunized as previously described (5) with either the highly purified human or bovine Factor VIII or material which had only been purified through the early stages of the procedure (up through the adsorption and elution from the triacelium citrate-cellulose column).

Electrophoresis—Polyacrylamide gel electrophoresis of the reduced Factor VIII was carried out by a modification of the general method of Weber and Osborn (15). In the present experiments, electrophoresis was performed in 0.2 m phosphate buffer, pH 7.0, containing 0.2% SDS. A 5% gel was employed and electrophoresis was carried out for 5 to 7 hours at room temperature. The gels were stained for protein with Coomassie blue. When staining for carbohydrate, the SDS was removed from the gels by dialysis for 24 hours against a solution containing 5% tri-o-chloroacetic acid and 5% v/v sodium dodecyl sulfate. The gels were then stained by the method of Zacharius et al. (16).

Zone electrophoresis was carried out on microscope slides precoated with 1 ml of 0.5% agarose in water and allowed to dry at 37°. The slides were then layered with 1.6 ml of 0.5% agarose solution containing 2% unpolymerized acrylamide and 0.05 M sodium barbital buffer, pH 8.6, as described by Williams and Chase (17). Samples (10 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 was performed on microscope slides (25 × 75 mm) coated with 0.5% agarose following the method of Scheidegger (18). 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 30 to 45 min. Antibody was added to the center trough and allowed to diffuse for at least 48 hours. The slides were then soaked in 0.15 M NaCl for at least 48 hours and then dried at 37°. They were subsequently stained for protein with Coomassie blue in methanol-acetic acid-water (5:1:5) for 5 min and destained in 7% acetic acid followed by a 95% ethanol wash.

Amino Acid Analysis—Samples for amino acid analysis were prepared by the method of Moore and Stein (19). Three samples (2 to 4 mg) were placed in 1 ml of 6 N constant boiling HCl, deoxygenated, and sealed in glass tubes. Hydrolysis was carried out for 24, 48, 72, and 96 hours at 110°. Amino acid analyses were carried out with a Spinco model 120 amino acid analyzer according to the method of Spackman et al. (20). Samples from two different preparations of Factor VIII were employed and gave essentially identical results. The reported values for serine and threonine were extrapolations to zero time hydrolysis, whereas isoleucine, leucine, and valine values were the average of the 96-hour hydrolyses. Half-cystine and methionine were determined after performic acid oxidation according to Hirs (21). Tryptophan was determined by the method of Beneze and Schmid (22). Free sulfhydryl groups were determined by the method of Ellman (23) after deamidation of the protein in 8 M urea.

Ultracentrifuge Analysis—Sedimentation equilibrium experiments were performed as described by Harris et al. (24) and Sercy et al. (25). Samples of human Factor VIII were prepared by dialyzing 2 to 4 mg of protein against 0.04 M Tris·HCl, pH 7.4, and 6 M guanidine at 25° for 12 to 14 days prior to each experiment. For subunit determinations, 0.04 M dithiothreitol was added to the above buffer and the flask flushed with nitrogen and sealed with a rubber stopper. This procedure avoided oxidation of the dithiothreitol and insured total reduction of the protein. In all experiments, the concentration in each of the three cells was 1.0, 0.7, and 0.5 mg per ml, respectively. Calculations were made by the methods discussed by Teller et al. (26) and Teller (27). The sedimentation equilibrium data were obtained using the short column, high speed technique of Yphantis (28). A six-channel Kcl-F counterpick was employed, and the interference patterns were recorded on Kodak II-G photographic plates. Rayleigh interference fringes were measured by a modified Nikon microcomparator automated as described by Del Rosier et al. (29). This method yields far more precise data than those collected manually. 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. (30).

To correct this value for 6% carbohydrate content (see Table IV), a value of 0.62 ml per g was em-
ployed (31). Thus, the actual value of $\delta = (0.74 \text{ ml per g} \times 0.94) + (0.62 \text{ ml per g} \times 0.06) = 0.73 \text{ ml per g}$. Other procedures and computations were performed as described (32). The SDS gel electrophoresis method of Weber and Osborn (15) was employed for estimating the molecular weight of Factor VIII subunits.

Clotting Assays—Platelet-deficient citrated plasma was prepared from normal human blood and patients with various coagulative abnormalities as previously described (32). In this procedure, plasma was not permitted to come in contact with glass and was frozen and stored at $-80^\circ$ until employed. Pooled platelet-deficient citrated human plasma was employed as a reference standard in the Factor VIII assay and was stored at $-80^\circ$.

Factor VIII activity was measured by a modification of the kaolin-activated partial thromboplastin time employing the methods of Landell et al. (33) and Proctor and Rapaport (34). In this assay, 0.1 ml of Factor VIII-deficient plasma (Factor VIII level less than 1%) was incubated for 10 min with 0.1 ml of an appropriate dilution of Factor VIII and 0.1 ml of a solution containing 0.05% Centrolex-P and 0.2% kaolin in 0.01 M Tris-HCl, pH 7.4, and 0.15 M NaCl. The Centrolex-P-kaolin solution was preincubated for 10 min at 37$^\circ$ prior to use. The assay mixture was then resealed by the addition of 0.1 ml of 0.033 M CaCl$_2$. The time required to form a visible clot after the addition of Ca$^{2+}$ was recorded with a stop watch. Further details of this assay are described elsewhere (5). One unit of Factor VIII was arbitrarily chosen as that amount of activity present in 1 ml of fresh human plasma.

Fibrinogen was assayed employing the methods of Jacobsson (36) and Claus (38). Various other coagulation factors (Factors VII, IX, X, and XII) and plasminogen were determined essentially as described by Biggs and Macfarlane (37).

Turnover Studies in Hemophilic Dogs—Studies of bovine and human Factor VIII turnover were performed in hemophilic beagles obtained from Dr. R. G. Buckner of Oklahoma State University. These animals weighed about 8 kg each. Ten minutes before the turnover experiments began, the dogs were given 20 mg of diphenhydramine hydrochloride intravenously. Ten milliliters of the hemophilic dog blood were then drawn and placed in a tube containing 1 ml of 4% sodium citrate. Factor VIII was then dissolved in 10 ml of sterile NaCl solution and slowly injected into a cephalic vein. Two milliliters samples were then removed by centrifugation at $4^\circ$ and 7000 x $g$ for 10 min. The supernatant was then discarded and the precipitate dissolved at 37$^\circ$ in 15 to 20 ml of 0.2 M Tris-HCl and 0.5% $\epsilon$-amino-$\nu$-caproic acid, pH 8.5, at a flow rate of 8 to 10 ml per min. The flow rate was reduced to 2.5 ml per min after 400 ml of effluent had passed through the column, and 25-ml fractions were then collected.

Fractions containing the majority of the Factor VIII activity (~220 ml) were mixed with 1/10 volume of 40% polyethylene glycol in 0.25 M NaCl and 0.2 M imidazole, pH 6.5. This solution was then placed in an ice-water bath for 10 min after which the precipitate was centrifuged at 4$^\circ$ and 7000 x $g$ for 10 min. The supernatant was then discarded and the precipitate dissolved in 15 to 20 ml of 0.2 M KCl and 0.02 M Tris-HCl, pH 7.4, at 37$^\circ$. Further concentration of the Factor VIII was accomplished by the addition of 1.8 ml of concanavalin A (25 mg per ml dissolved in 1.0 M NaCl). The solution was mixed and then allowed to stand at room temperature for 10 min. The flocculent precipitate was centrifuged at room temperature for 5 min at 2000 x $g$ and then dissolved at 37$^\circ$ in 2 ml of 0.25 M NaCl-0.02 M imidazole (pH 6.5) containing 10% methyl-$\alpha$-$\nu$-glucopyranoside (B grade) and 0.03% sodium azide and allowed to stand overnight at 4$^\circ$.

The final step in the purification was passage of the concanavalin A-glycoprotein solution (2.5 to 3 ml) through a column (1.5 x 100 cm) of A-15m agarose pre-equilibrated with the same methyl-$\alpha$-$\nu$-glucopyranoside buffer solution as above. Elution
of purified Factor VIII was performed at a rate of 15 ml per hour, and 3-ml samples were collected after 40 ml had passed through the column. The contents of the first four tubes, containing approximately 75% of the Factor VIII activity, were combined and the Factor VIII was precipitated with 10 volume of 40% polyethylene glycol (15 min at 0°) followed by centrifugation at 16,000 × g for 10 min at 4°. The Factor VIII precipitate was dissolved in 2.0 ml of 0.01 M Tris-HCl, pH 7.4, 0.015 M NaCl, and 0.03 M sodium azide and stored at 4°. Under these conditions, human Factor VIII activity was stable for about 2 weeks; after 4 weeks, the activity drops approximately 50%. When quick-frozen and stored at −80°, the stability was greatly enhanced.

The yield from the agarose gel filtration step improves markedly after the column has been used three or four times. The column can then be used another 25 to 30 times if it is washed with 600 to 800 ml of the imidazole buffer containing methyl-α-D-glucopyranoside prior to reutilization. The yield for this step can also be increased by including those tubes on the ascending portion of the first peak (see lower panel, Fig. 1). These preparations, however, contain trace amounts of fibrinogen which can be detected by disc gel electrophoresis. In some Factor VIII preparations, all of the tubes in the first peak which contained Factor VIII activity were combined and the fibrinogen in these preparations was removed by passing the solution through an agarose column to which human fibrinogen antibody was covalently bound (43, 44).

In more recent experiments, the Al(OH)₃ adsorbed cryoprecipitate solution was stirred for 15 min at 20° with 150 g of prewashed calcium citrate in 250 ml of 0.05 M citrate buffer. The slurry was then centrifuged for 5 min at 7000 × g at 16° and the calcium citrate then washed two more times with 500 ml of citrate buffer and centrifuged at 4°. The calcium citrate precipitate was then mixed with 1 liter of citrate buffer containing 150 g of cellulose powder and the mixture placed in a polypropylene column (7.5 × 30 cm) at 4°. The column was allowed to settle and the Factor VIII was eluted as described above. This modification speeds up the purification procedure by 1 to 2 hours.

**RESULTS**

**Preparation of Human Factor VIII**—The purification steps for human Factor VIII and the yield for a typical preparation are shown in Table I. The cryoprecipitate employed contained Factor VIII purified approximately 10- to 20-fold with a recovery of about 30%. The starting material varied, however, from 5- to 20-fold in purification and 25 to 45% in yield from plasma. Residual amounts of Factors VII, IX, and X and prothrombin activity were removed from the cryoprecipitate by an Al(OH)₃ adsorption step, and the protein was then subjected to calcium citrate-cellulose column chromatography (top panel, Fig. 1). A major portion of the protein applied to the column appeared in the solvent front and contained approximately 10% of the Factor VIII activity, while the adsorbed Factor VIII was eluted in the second protein peak. The final step involved precipitation of Factor VIII by concanavalin A and subsequent gel filtration on agarose A-15m (lower panel, Fig. 1). Factor VIII activity appeared in the breakthrough peak from the agarose column which separated it from concanavalin A and the remaining traces of fibrinogen as well as other contaminating proteins.

**Fig. 1.** Calcium citrate-cellulose column chromatography of an Al(OH)₃ adsorbed cryoprecipitate (top panel) and gel filtration of partially purified human Factor VIII on agarose A-15m (bottom panel). The adsorbed cryoprecipitate (140 ml in 0.05 M sodium citrate, 0.5% ε-amino-n-caproate acid, pH 6.5) was applied to the calcium citrate-cellulose column and eluted as described under “Methods.” Similarly, 2 to 3 ml of the concanavalin A precipitated Factor VIII were eluted from the agarose A-15m column with 0.25 M NaCl-0.02 M imidazole buffer, pH 6.5, containing 10% methyl-α-D-glucopyranoside and 0.03 M sodium azide. □--□, protein concentration as determined by absorbance at 280 nm; □--□, Factor VIII activity.

**Table I**

Purification of human Factor VIII from cryoprecipitate

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein mg</th>
<th>Total units</th>
<th>Specific activity units/mg</th>
<th>Yield</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryoprecipitate</td>
<td>11,600</td>
<td>750</td>
<td>0.065</td>
<td>1</td>
<td>10-20</td>
</tr>
<tr>
<td>Al(OH)₃</td>
<td>9,990</td>
<td>750</td>
<td>0.075</td>
<td>100</td>
<td>1-1.1</td>
</tr>
<tr>
<td>Calcium citrate-cellulose</td>
<td>96</td>
<td>260</td>
<td>2.5</td>
<td>34.7</td>
<td>38.5</td>
</tr>
<tr>
<td>Concanavalin A-agarose filtration</td>
<td>5</td>
<td>170</td>
<td>34</td>
<td>22.6</td>
<td>523</td>
</tr>
</tbody>
</table>
Factor VIII, and reduced and alkylated Factor VIII. Factor VIII (10 μl, containing 10 to 20 μg of protein) was added to each of the wells and electrophoresis carried out as described under "Methods." Sample 1 is native Factor VIII, Sample 2 is reduced Factor VIII, and Sample 3 is reduced and alkylated Factor VIII.

**FIG. 2.** Zone electrophoresis of human Factor VIII, reduced Factor VIII, and reduced and alkylated Factor VIII. Factor VIII (10 μl, containing 10 to 20 μg of protein) was added to each of the wells and electrophoresis carried out as described under "Methods." Sample 1 is native Factor VIII, Sample 2 is reduced Factor VIII, and Sample 3 is reduced and alkylated Factor VIII.

Completion of the electrophoresis, 50 μl of the appropriate rabbit antibody solution were applied to the center trough. Wells 1 and 2 represent two different Factor VIII preparations (10 μl containing 10 to 15 μg of protein) which were precipitated by a rabbit antibody as employed in the top slide. Well 5 contained 40 μg of highly purified human Factor VIII, while Well 6 contained 50 μg of protein from the calcium citrate column, and the center trough contained the same antibody as employed in the top slide. Well 7 contained 45 μg of protein from the calcium citrate column, while Well 8 contained 20 μg of highly purified Factor VIII and the center trough contained rabbit antibody against those proteins present in the calcium citrate eluate. Well 7 contained 45 μg of 99% clottable human fibrinogen, and Well 8 contained 20 μg of highly purified human Factor VIII. The center trough contained an antibody prepared against partially purified Factor VIII. The anode was at the right in all slides and protein migration was from left to right.

**FIG. 3.** Immunoelectrophoresis of highly purified human Factor VIII, partially purified human Factor VIII, and highly purified human fibrinogen. Immunoelectrophoresis was carried out for 75 min as described under "Methods." The antibodies employed were also prepared as described under "Methods." Various protein solutions were placed into the appropriate well, and electrophoresis performed as described under "Methods." Upon completion of the electrophoresis, 50 μl of the appropriate rabbit antibody solution were applied to the center trough. Wells 1 and 2 represent two different Factor VIII preparations (10 μl containing 10 to 15 μg of protein) which were precipitated by a rabbit antibody to highly purified Factor VIII and stained for protein with Coomassie blue. Well 3 contained 15 μg of highly purified human Factor VIII, while Well 4 contained 25 μg of protein from the calcium citrate column, and the center trough contained the same antibody as employed in the top slide. Well 5 contained 40 μg of protein from the calcium citrate column, while Well 6 contained 40 μg of highly purified Factor VIII and the center trough contained rabbit antibody against those proteins present in the calcium citrate eluate. Well 7 contained 45 μg of 99% clottable human fibrinogen, and Well 8 contained 20 μg of highly purified human Factor VIII. The center trough contained an antibody prepared against partially purified Factor VIII. The anode was at the right in all slides and protein migration was from left to right.


Increasing the mobility of the Factor VIII relative to the reduced or native Factor VIII.

**Immunoelectrophoresis**—As another criterion for purity, human Factor VIII was subjected to immunoelectrophoresis on agarose slides (Fig. 3). The antibody employed in these experiments was prepared in rabbits against highly purified Factor VIII and a partially purified Factor VIII preparation which contained fibrinogen. These antibodies, as shown later, readily inhibit Factor VIII activity. In Samples 1 and 2, the highly purified Factor VIII was subjected to electrophoresis followed by immunodiffusion against rabbit antibody to highly purified Factor VIII. After staining with Coomassie blue, single sharp precipitin lines were obtained.

The antibody prepared against highly purified Factor VIII also yields a single precipitin line with partially purified Factor VIII. The pattern shown in Sample 3 contains highly purified Factor VIII, while Sample 4 contains partially purified Factor VIII. In Sample 5, a partially purified Factor VIII preparation as well as an antibody against partially purified Factor VIII were employed. Many precipitin lines are obtained with the partially purified preparation of Factor VIII; however, the antibody yields only a single precipitin line with the highly purified Factor VIII (Sample 6), indicating removal of many contaminating proteins during the final purification. Sample 7 shows the precipitin line for fibrinogen with an antibody to partially purified Factor VIII, while Sample 8 contains only the highly purified Factor VIII. The precipitin lines shown in these experiments were identical with those observed by indirect lighting, indicating that the staining procedure was detecting all of the precipitin lines present.

**Polyacrylamide Gel Electrophoresis of Human Factor VIII**—Human Factor VIII, like bovine Factor VIII (5), did not enter a 3.25% polyacrylamide gel even in the presence of 8 M urea. After reduction, a single protein band was obtained in 5% polyacrylamide gels in the presence of SDS. These experiments, shown in Fig. 4, were performed with 20, 30, and 50 μg samples of protein (Samples 1, 2, and 3, respectively). Sample 4 contained an equal amount of reduced human Factor VIII and a highly purified sample of reduced bovine Factor VIII. Sample 5 contained only reduced bovine Factor VIII. The results of these experiments support the idea that human Factor VIII is made up of subunits which are similar or perhaps identical, and that these subunits are held together by disulfide bonds. Furthermore, the subunits in human as well as bovine Factor VIII appear to be essentially the same size.

The subunits in human Factor VIII contain carbohydrate, as shown in the SDS polyacrylamide gel electrophoresis patterns in Fig. 5. In these experiments, the protein was reduced with 0.1 M 2-mercaptoethanol. The gel on the right was stained for protein with Coomassie blue, and the gel on the left for carbohydrate with the periodic acid-Schiff reagent. A single protein and a single carbohydrate band running in the same position was obtained for each sample. These data indicate that human Factor VIII, like bovine Factor VIII, is composed of glycoprotein subunits.

The subunits of human Factor VIII migrate slower in SDS gel electrophoresis than the α, β, and γ subunits of fibrinogen, which have molecular weights of 47,000, 56,000, and 70,900, respectively (45). The molecular weight estimated for reduced Factor VIII by the SDS method was 240,000.

**Ultracentrifugation of Human Factor VIII**—Sedimentation equilibrium studies of human Factor VIII indicated that the protein was homogeneous at all protein concentrations tested. The molecular weight was calculated to be 1.12 million ± 98,000...
methionine, tyrosine, and tryptophan content. Neither human nor bovine Factor VIII was found to contain free sulfhydryl groups.

The carbohydrate compositions for human Factor VIII and that previously determined for bovine Factor VIII (5) are presented in Table IV. The human preparation contains approximately 6% carbohydrate, including 2.2% hexose, 2.7% hexosamine, and 0.9% neuraminic acid. In contrast, bovine Factor VIII contains approximately 20% carbohydrate. Also, there are approximately equal amounts of hexose and hexosamine in human Factor VIII, while bovine Factor VIII contains approximately twice as much hexose as hexosamine.

**Chemical Composition of Human Factor VIII**—The amino acid composition for human Factor VIII is shown in Table III along with the amino acid composition for bovine Factor VIII (5). It is clear that the amino acid contents of the two proteins are considerably different. Both, however, have a relatively low

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\eta_m$</th>
<th>$\bar{\eta}$</th>
<th>$M_w$</th>
<th>$M_x$</th>
<th>$M_t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denatured</td>
<td>5,090</td>
<td>0.733</td>
<td>1.120 ± 0.09</td>
<td>1.210 ± 0.101</td>
<td>1.270 ± 0.101</td>
</tr>
<tr>
<td>Denatured and reduced in dithiothreitol</td>
<td>14,000</td>
<td>0.733</td>
<td>138 ± 8</td>
<td>194 ± 15</td>
<td>244 ± 27</td>
</tr>
</tbody>
</table>

*These values represent an average of the values obtained for the whole cell average molecular weights at the three different protein concentrations employed as described under “Methods.”

* $M_t$ refers to the smallest species which could be calculated by the methods described by Teller et al. (26) and Teller (27). The uncertainties are weighted root mean square values in this column, but standard deviations for all other values.

* Buffer: 0.04 M Tris-HCl, 6.0 M guanidine HCl, pH 7.5.

* Buffer: same as c with the addition of 0.04 M dithiothreitol.

![Image of SDS gel electrophoresis](http://www.jbc.org/)

**Fig. 4.** SDS polyacrylamide gel electrophoresis of highly purified reduced human and reduced bovine Factor VIII. From left to right, Gels 1, 2, and 3 contained 20, 30, and 50 pg of purified human Factor VIII, respectively. Gel 4 represents 50 pg of a mixture of equal amounts of human and bovine Factor VIII, while Gel 5 contained 50 pg of bovine Factor VIII alone. The protein on these gels was stained with Coomassie blue. The anode was at the bottom of the gel.

**Fig. 5.** SDS polyacrylamide gel electrophoresis of reduced human Factor VIII stained for protein or carbohydrate. Both gels contain 75 pg of Factor VIII. The gel on the left was stained for carbohydrate, while the gel on the right was stained for protein as described under “Methods.” The anode was at the bottom of the gel.
Table III
Amino acid composition of human and bovine Factor VIII

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Human Factor VIII</th>
<th>Bovine Factor VIII</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>residues/10^6 g</td>
<td>proteinа</td>
</tr>
<tr>
<td>Lysine</td>
<td>39.9</td>
<td>53.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>22.5</td>
<td>22.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>39.9</td>
<td>45.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>85.4</td>
<td>85.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>60.1</td>
<td>43.8</td>
</tr>
<tr>
<td>Serine</td>
<td>70.8</td>
<td>57.5</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>89.9</td>
<td>84.4</td>
</tr>
<tr>
<td>Proline</td>
<td>61.2</td>
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</tr>
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<td>Glycine</td>
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</tr>
<tr>
<td>Alanine</td>
<td>63.8</td>
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<td>Half-cystineб</td>
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<td>Valine</td>
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<td>Isoleucine</td>
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<td>Tyrosine</td>
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<tr>
<td>Phenylalanine</td>
<td>27.0</td>
<td>26.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>24.7</td>
<td>17.5</td>
</tr>
</tbody>
</table>

а The amino acid composition is expressed in residues per 10^6 g of protein (not glycoprotein).
б Determined as cysteic acid.
в Determined by the spectrophotometric assay.

Table IV
Carbohydrate composition of human and bovine Factor VIII

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Human Factor VIII</th>
<th>Bovine Factor VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>residues/100,000 g</td>
<td>%</td>
</tr>
<tr>
<td>Hexose</td>
<td>12.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>15.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Neuraminic acid</td>
<td>2.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Total</td>
<td>5.8</td>
<td>19.6</td>
</tr>
</tbody>
</table>

а The carbohydrate content is expressed in residues per 10^6 g of glycoprotein (not protein).

Then diluted 300- to 500-fold and an aliquot assayed in Factor VIII-deficient plasma. The solid curve on the left is the neutralization of human Factor VIII by antibody prepared against highly purified human Factor VIII, and the solid curve on the right is the neutralization of human Factor VIII by antibody prepared against bovine Factor VIII. These experiments indicate that the antibodies prepared against either human or bovine Factor VIII inactivate human Factor VIII. Approximately 20 times more antibody to bovine Factor VIII, however, was required to cause 50% inhibition of human Factor VIII than that required to cause the same inhibition with bovine Factor VIII (dashed curve on left). Similarly, antibodies to either human or bovine Factor VIII neutralize bovine Factor VIII. Those experiments are represented by the dashed curves. The dashed curve on the left represents bovine Factor VIII versus antibody to bovine Factor VIII, and the dashed curve on the right is bovine Factor VIII versus antibody to human Factor VIII. From these experiments, one can conclude that bovine Factor VIII was inhibited by antibodies to both human and bovine Factor VIII. Furthermore, approximately 20 times more antibody to human Factor VIII was required to neutralize bovine Factor VIII than that required for human Factor VIII. Thus, the antibodies pre-

Fig. 6. Neutralization of human and bovine Factor VIII activity with either rabbit anti-human or anti-bovine Factor VIII antibodies. The two curves on the left represent the homologous system; i.e., human Factor VIII versus antibody to human Factor VIII (○—○), and bovine Factor VIII versus antibody to bovine Factor VIII (○—○). Conversely, the two curves on the right represent the heterologous system; i.e., human Factor VIII versus antibody to bovine Factor VIII (○—○), and bovine Factor VIII versus antibody to human Factor VIII (○—○). Samples were incubated and assayed as described in the text and under "Methods."pared against Factor VIII are approximately 20 times more potent as inhibitors toward the homologous protein as compared to the heterologous molecule.

Immunoelectrophoresis of Cryoprecipitate from Normal and Hemophilic Plasma—With an antibody prepared in rabbits from a highly purified human Factor VIII, it was possible to test for the presence of cross-reactive material in the cryoprecipitate of an individual with classic hemophilia. Immunoelectrophoresis experiments employing highly purified Factor VIII and a cryoprecipitate from pooled normal plasma and from Factor VIII-deficient plasma are shown in Fig. 7. Single sharp precipitin lines were obtained with pure Factor VIII (Sample 1 and 2), normal cryoprecipitate (Sample 3), and cryoprecipitate from a patient with classic hemophilia (Sample 4).

Turnover of Factor VIII in Hemophilic Dogs—The final proof of identity of a Factor VIII preparation is evidence that it will elevate the plasma level of Factor VIII in an individual with classic hemophilia. For our initial studies, hemophilic dogs were employed since human or bovine Factor VIII readily corrects hemophilic dog plasma in vitro. Highly purified human Factor VIII (280 units) was given by intravenous injection, and samples were removed from the hemophilic dogs during the next 18 hours. The initial recovery of Factor VIII was approximately 56% of the infused Factor VIII assuming a blood volume of 80 ml per kg of body weight (46). The half-life in vivo for human Factor VIII was approximately 4 hours. In similar experiments with highly purified bovine Factor VIII (360 units), the initial recovery was approximately 55% and the half-life in vivo was 9 hours. It is
clear that either human or bovine Factor VIII corrects the clotting defect in the plasma of hemophilic dogs, and the isolation of these proteins has not resulted in their partial degradation leading to an abnormally rapid clearance. A half-life of 4 to 9 hours of human or bovine Factor VIII in hemophilic dogs is similar to that obtained for human Factor VIII in patients with classic hemophilia (47, 48).

**DISCUSSION**

Human Factor VIII, like bovine Factor VIII, is a high molecular weight glycoprotein. The carbohydrate level in human Factor VIII is approximately 6%, which is substantially lower than that found in bovine Factor VIII (Table IV). The carbohydrate content of human Factor VIII found in the present experiments is very similar to that recently reported by Marchesi et al. (7) and by McKee (53), but it is lower than the value of 10% reported by Herzigold et al. (6). In preliminary experiments, it was found that human Factor VIII prepared by the present method contains approximately 5% lipid. Other investigators have also noted the presence of lipid in human Factor VIII (6, 7). The lipid may be a contaminant, however, since Ratnoff et al. (54) found that the lipid content of human Factor VIII varied depending on the chylomicron content of the starting plasma. No lipid was found in the highly purified bovine Factor VIII (5).

The amino acid compositions of bovine and human Factor VIII are different as shown in Table III. Some immunological cross-reactivity, however, exists between these two proteins. Antibodies prepared against each of these two highly purified proteins will inactivate Factor VIII from either human or bovine sources. The potency of the respective antibodies against the homologous protein, however, is much greater than that toward the heterologous protein. These experiments suggest that there may be large regions in the human and bovine Factor VIII molecule which differ, but active site regions important for biological activity may be very similar or perhaps identical in both proteins. Cross-reactivity of human or bovine Factor VIII with antiserum to crude bovine or human Factor VIII has also been studied by Denson (55) who found similar results.

With an antibody prepared against highly purified human Factor VIII, it was also possible to demonstrate the presence of an inactive cross-reactive Factor VIII molecule present in the
cryoprecipitate of an individual with classic hemophilia. These experiments, which have been carried out with only one individual with classic hemophilia, confirm the results published by other investigators (36-60) who have reported the presence of an abnormal Factor VIII protein in individuals with less than 1% Factor VIII activity. The present data, however, are the first to employ an antibody prepared against highly purified Factor VIII with well defined physicochemical properties. These data are consistent with the hypothesis that an abnormal Factor VIII molecule is synthesized in individuals with classic hemophilia. Furthermore, this protein is probably inactive due to an amino acid replacement in some essential portion of the molecule. It also appears probable that several classes of abnormal Factor VIII molecules will be found in individuals with classic hemophilia, but further investigations will be required to test this possibility.

The molecular weight of human Factor VIII, determined by sedimentation equilibrium experiments, was 1.12 million ± 98,000. In these experiments, the intact protein was homogeneous at all concentrations tested. This value is very similar to that found for the bovine Factor VIII, although this protein was found to be heterogeneous in sedimentation equilibrium experiments (5). In sedimentation velocity experiments, it was found that human Factor VIII has an $s_{20,w}$ value of 23.7 ± 0.5 when examined in 0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.05 M $\epsilon$-amino-$n$-caproic acid. This value is consistent with that expected for a large macromolecule. In the present studies, there were no indications of active Factor VIII molecules of smaller molecular weight. Similar results were previously obtained for bovine Factor VIII (5).

The molecular weight of the subunit of human Factor VIII remains unclear. Sedimentation equilibrium studies of the subunits formed in the presence of diithiothreitol indicate that the preparation was heterogeneous. The molecular weight of the smallest species was calculated as 105,000 ± 5,000. It appears likely that considerable aggregation of similar or identical subunits was taking place during these studies, however, since the reduced Factor VIII gave only one band in polyacrylamide gel electrophoresis and zone electrophoresis. The molecular weight determined by SDS gel electrophoresis is very large compared to that calculated from sedimentation equilibrium experiments. Similar results have been obtained by others (7, 53). The molecular weight determined by SDS gel electrophoresis may be high, however, due to the presence of carbohydrate in Factor VIII. High molecular weights of glycoproteins have been noted by others (61, 62) who have found an anomalous electrophoretic behavior of the glycoproteins isolated from erythrocyte membranes.

Highly purified human Factor VIII, like bovine Factor VIII, readily undergoes thrombin activation. Whether thrombin modification of Factor VIII and perhaps Factor V is related to a hypercoagulable state is unclear at the present time. Thrombin modification of Factor VIII in vitro is most likely under strict regulatory control which may be abnormal in patients suffering from hypercoagulability. The molecular events associated with the thrombin modification of Factor VIII are presently under investigation in our laboratory.

Acknowledgments—We wish to express our sincere thanks to Drs. Robert Meyer and Kazuo Fujikawa for valuable discussions, to Dr. David Teller for his assistance in the sedimentation equilibrium experiments, and to Charles Nicholas and Richard Granberg for excellent technical assistance. We are also indebted to the King County Blood Bank and Dr. Dennis Donohue for the human cryoprecipitate which made these studies possible.

REFERENCES


Isolation and Characterization of Human Factor VIII (Antihemophilic Factor)
Mark E. Legaz, Gottfried Schmer, Richard B. Counts and Earl W. Davie