Studies of the Human Factor VIII/von Willebrand's Factor Protein. II. Identification and Characterization of the von Willebrand Protein

By Harvey R. Gralnick and Barry S. Coller

The purified factor VIII-related protein we have previously characterized from normal cryoprecipitate possesses both procoagulant activity and vWF activity. We have attempted to isolate and characterize this protein from three patients with severe vWD. This protein is absent or markedly diminished in amount in these vWD patients, as judged by gel filtration, polyacrylamide-gel electrophoresis, and immunoprecipitation assays. Likewise, the procoagulant and vWF activities are deficient. As vWF activity is one of the major biologic functions of either the normal or hemophilic factor VIII-related protein, the purified protein should be designated the f VIII/vWF protein.

The human factor VIII/von Willebrand factor (f VIII/vWF) protein in normals and hemophilia A patients has been characterized as a high-molecular-weight glycoprotein with a major subunit of 195,000-240,000 daltons. Immunologic studies of normal and hemophilia A plasmas or plasma concentrates have revealed normal or increased levels of the f VIII/vWF antigen, while in most von Willebrand's disease (vWD) materials this antigen is reduced or absent. Two biologic functions have been attributed to the normal protein in vitro: (1) procoagulant activity: the ability to correct the abnormal coagulation time of hemophilia A plasma, and (2) von Willebrand factor activity: the ability to correct either the reduced platelet retention or ristocetin-induced platelet aggregation found in vWD. Both of these biologic activities have been found in normal cryoprecipitate and the void volume fraction(s) of gel-filtered normal cryoprecipitate. We have purified the factor VIII/vWF protein with chymotrypsin-digested cryoprecipitate by a one-stage gel chromatography procedure. In the present study, we characterized and tested the in vitro biologic activities of this purified void volume protein and other column fractions prepared from normal, hemophilic, and vWD cryoprecipitates. Gel chromatography, polyacrylamide-gel electrophoresis, and immunologic assays allowed us to correlate the functional, immunologic, and biochemical properties of the protein.

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Abbreviations: SDS, sodium dodecyl sulfate; vW, von Willebrand's; vWD, von Willebrand's disease; vWF, von Willebrand's factor; f VIII/vWF, factor VIII/von Willebrand's factor. The term factor VIII/von Willebrand factor denotes the purified material from normal cryoprecipitate which (1) corrects the coagulation defect in hemophilic plasma and (2) corrects the defects in platelet retention in a glass-bead column and ristocetin-induced aggregation of von Willebrand's disease platelets.

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MATERIALS AND METHODS

Cryoprecipitate and Plasma

Cryoprecipitate was prepared from the plasma obtained from a 1-U plasmapheresis performed on 13 normal individuals, two patients with hemophilia A, and three patients with vWD. The blood was collected in plastic bags containing 1 volume 4% sodium citrate (Fenwal Laboratories, Morton Grove, Ill.), and after centrifugation at 5000 g at 4°C for 15 min, the plasma was removed and respun. The cell-free plasma (240 ± 20 ml/U) was then frozen at −30°C. The cryoprecipitate was prepared in the smallest volume possible and brought to a total of 11-12 ml by the addition of supernatant plasma. All cryoprecipitates were prepared and used immediately. Hyland Method IV was prepared and reconstituted as previously described. Whole blood (30-40 ml) was collected in nonwettable tubes in 1 volume 40% sodium citrate. Cell-free plasma was obtained and stored as above. In experiments with plasma, 10-ml aliquots were thawed at 37°C and used immediately for gel-filtration experiments.

Patients

The normal individuals were volunteer blood donors. The two patients with hemophilia A were males who had family histories of an X-linked bleeding disorder with factor VIII levels varying between less than 0.01 U/ml (1%) to 0.02 U/ml (2%). The three patients with vWD had abnormal platelet retention, prolonged bleeding times, low factor VIII levels, and absent antigen levels during several months of observation. One patient, vW I, is a female with 0.03 U/ml (3%) factor VIII, a bleeding time of greater than 30 min, 0%-5% platelet retention in a glass-bead column (normal > 90%), and no family history of hemophilia. Patient vW II is a female with a factor VIII of 0.04 U/ml (4%), a bleeding time greater than 30 min, and platelet retention of 5%. The third patient, vW III, is a male with a factor VIII level of 0.05 U/ml (5%), a bleeding time of > 30 min, and platelet retention of 10%. Each vWD patient was studied twice. The patients did not receive plasma or plasma concentrates for 6 mo prior to this study.

Alpha-chymotrypsin Digestion

Alpha-chymotrypsin (Worthington Biochemical Corp., Freehold, N.J.) from bovine pancreas was dissolved in 0.001 M hydrochloric acid at concentrations of 10 mg/ml just before use. The cryoprecipitate was digested at 24°C. Digestion was initiated with 0.25 ml of chymotrypsin, and then further 0.03-ml additions were made at 15-30-min intervals until digestion was completed as determined by prolongation of the thrombin time above 300 sec. The digest mixtures were then immediately placed at 4°C, centrifuged twice at 27,000 g, and then supernatant was applied to the column. Digestion of cryoprecipitate was completed within 180-210 min. Factor VIII levels were measured before and at the end of the digestion.

Coagulation Assays

All coagulation tests were performed on a fibrometer (BioQuest Division, Cockeysville, Md.). All factor VIII determinations were performed by a one-stage assay (partial thromboplastin time) using hemophilic plasma (< 1% factor VIII activity) as substrate. Units of factor VIII activity were calculated by defining 1 U of factor VIII activity as the amount contained in 1 ml of lyophilized plasma (AHG-PTC-PTA Reference Plasma, Hyland Laboratories, Costa Mesa, Calif.). Thrombin clotting times to monitor the alpha-chymotrypsin digestion were performed by adding 0.1 ml of the digestion mixture or undigested cryoprecipitate to 0.2 ml (2.5 U/ml) bovine thrombin solution (Parke-Davis & Co., Detroit, Mich.) at 37°C and measuring the time for clotting to occur. The thrombin time on undigested cryoprecipitate varied between 24 and 27 sec.

Gel Filtration

Gel filtration of the digested cryoprecipitate was performed on a 2.5 × 40-cm Pharmacia column packed with Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). Ten milliliters of undigested cell-free plasma(s) were chromatographed on the same column. The void volume was determined with dextran blue 2000 (Pharmacia Fine Chemicals), fetal calf thymus DNA (Sigma...
Chemical Co., St. Louis, Mo.), and lipopolysaccharide from Salmonella typhosa 0901 (Difco Laboratories, Detroit, Mich.). Columns were eluted at 4°C at a pressure of approximately 20 cm H$_2$O with buffer containing 0.05 M Tris (Mann ultra-pure) and 0.1 M sodium chloride adjusted with hydrochloric acid to pH 7.35. The flow rate of the column averaged 14 ml/hr, and 1.6-1.8-ml fractions were collected. Protein was monitored by absorbance at 280 nm. Protein concentration was measured by the method of Lowry, Rosebrough, Farr, and Randall.$^{12}$

The void volume ($V_0$) of the column was 48 ml. Identical fractions were collected and concentrated from all column eluates: fraction 1, 48-61 ml; fraction 2, 62-75 ml; fraction 3, 76-89 ml; fraction 4, 90-103 ml; and fraction 5, 104-117 ml.

The column fractions were pooled for testing by polyacrylamide-gel electrophoresis, the ristocetin-induced platelet aggregation assay, or counterimmunoelectrophoresis. When necessary, normal and hemophilic column fractions were concentrated to 25%-50% of the original volume by dialysis against powdered Ficoll (Pharmacia Fine Chemicals) at 4°C. The vWd column fractions were concentrated to 10%-25% of the original volume. All column fractions were tested for vWF activity the same day or frozen at -30°C and tested within 7 days.

Preparation of Antibody to Factor VIII

Antibody was developed in New Zealand rabbits and Nubian goats to purified f.VIII/vWF prepared from Hyland Method IV.$^3$ Crystallized rabbit albumin (at a final concentration of 5 mg/ml; Miles Laboratories, Kankakee, Ill.) was added to the $V_0$ fractions prior to concentration to stabilize the procoagulant activity. The rabbits were immunized at weekly intervals by three subcutaneous injections of purified protein (0.86-1.0 mg per injection) suspended in incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich.), and they then received three booster immunizations 1 mo apart. The goats were immunized with three weekly intramuscular injections (average of 2 mg per dose) of purified protein without adjuvant. They then received booster immunizations once a month for 4 mo. The rabbits were bled by cardiac puncture and the goats by jugular venipuncture.

Blood from the animals was allowed to clot at 37°C for 4 hr. The serum was separated by centrifugation at 5000 g for 20 min and then heated to 56°C for 30 min. The heated serum was resup at 3000 g for 30 min and the supernatant material removed, divided into aliquots, and frozen at -60°C. The supernatant serum was adsorbed with either (1) an 8% ethanol precipitate of plasma from which a 3% ethanol precipitate had been removed$^7$ plus the same crystallized rabbit albumin (Miles Laboratories, Kankakee, Ill.) used to stabilize the procoagulant activity or (2) the supernatant plasma from the cryoprecipitate of a patient with severe vWd (vW I).

Counterimmunoelectrophoresis

Counterimmunoelectrophoresis was performed on lantern slides 3½ x 4 inches which were precoated with 1 ml of melted 1% agarose in barbital buffer, 0.025 M, pH 8.6. Sixteen milliliters of 1%, agarose dissolved in the same buffer were then applied to the plate. The plate was made by cutting antigen wells with a number 3 cork bore to contain 0.04 ml of material. Antibody wells were cut to contain 0.006 ml. The antigen and antibody wells were separated by 3 mm. After the application of antigen and antibody (goat or rabbit antihuman f.VIII/vWF), constant current (30 mA) was supplied for 90 min at 4°C using a 0.05 M barbital, pH 8.6, buffer in the troughs. After electrophoresis, the plate was read immediately, washed in 0.85% sodium chloride for 48 hr and in distilled water for 24 hr, dried and stained with 0.5% Amido-schwartz, and then read again. Relative quantitation of antigen was obtained by comparing the reactivity of dilutions of the test material (made in 0.05 M barbital buffer) with the reactivity of normal plasma. A pool of 30 normal blood donor plasmas and a commercially available pool of lyophilized plasma (AHF-PTC-PTA Reference Plasma, Hyland Laboratories, Costa Mesa, Calif.) were used as controls. The highest dilution of the lyophilized normal plasma which reacted was arbitrarily designated 100%, and all relative percentages were derived from comparison with this normal plasma. All hemophilic and vWd column fractions were analyzed with comparable normal material. Specific antigenic activity (antigen per unit protein) was defined as the highest reaction dilution of a column fraction divided by that fraction's absorbance at 280 nm.
Acrylamide-gel Electrophoresis

Acrylamide-gel electrophoresis was performed using the reagents and methods described by Canalco, Inc., Rockville, Md. Electrophoresis was performed in 5% (5.13 T, 2.5% C) acrylamide gels containing 0.1% SDS (Pierce Chemical Co., Rockford, Ill.), according to Shapiro, Vinuela, and Maizel\(^{13}\) and as previously described.\(^{5}\) The column pools (50–100 µg) were incubated for 3 hr at 37°C in 1% SDS-0.1 M sodium phosphate buffer, pH 7.1, containing 0.025% sodium azide with or without the addition of the reducing agent dithioerythritol (Mann Research, Orangeburg, N.Y.). Proteins used as standards were those previously described\(^{5}\) and, in addition, rabbit muscle myosin (194,000 daltons).

Platelet Aggregation With Ristocetin

Ristocetin-induced platelet aggregation studies on citrated (1/100 volume 40% citrate) platelet-rich plasma (PRP) adjusted to 300,000 platelets per cu mm were performed in a Chrono-log Aggregometer as originally described by Born and Cross.\(^{14}\) Ristocetin was supplied by Abbott Laboratories, Chicago, Ill.

Samples were tested for vWF activity (i.e., the ability to correct ristocetin-induced platelet aggregation) in one of two ways:

- A 50-µl aliquot of the test material was added to 0.4 ml of PRP from a patient with severe vWD (who had no response to ristocetin at a final concentration of 1.8 mg/ml), and after a 1-min incubation, 15 µl of ristocetin (50 mg/ml dissolved in 0.15 M NaCl, 0.01 M barbital, pH 7.4) was added (final concentration 1.6 mg/ml).
- Normal platelets were treated in one of two ways (see below) so that they no longer aggregated in response to ristocetin at the dose tested. This assay consisted of the addition of a 50-µl aliquot of the test material to 0.4 ml of the washed platelets, and after a 1-min incubation, 10–20 µl of ristocetin (50 mg/ml) was added (1.1–2.1 mg/ml final concentration).

Normal platelets were made unresponsive to ristocetin by washing or by gel filtration followed by washing. In the first instance, washing was performed by the technique of Zimmerman.\(^{15}\) Fifty milliliters of fresh whole blood were collected in 1/10 volume of acid-citrate dextrose and centrifuged at 1800 g and 25°C for 20 min. The platelet-poor plasma was removed and measured and replaced with an equal volume of a modified Tyrode's buffer. The cells and platelets were gently resuspended and centrifuged again. This procedure was repeated four times with the last centrifugation performed at 700 g and the platelet-rich buffer supernatant removed. A final centrifugation at 1200 g for 1 min reduced the red blood cell contamination. Platelet-rich buffer was kept at room temperature and used immediately. The modified Tyrode's buffer contained 2.0% bovine serum albumin (Pentex, Sigma Laboratories, St. Louis, Mo.) and 0.03 M adenosine (Sigma Laboratories, St. Louis, Mo.). The assay procedure consisted of adding 50 µl of test material to 0.4 ml of the platelet-rich buffer and then adding 20 µl (50 mg/ml) of ristocetin (final concentration 2.1 mg/ml).

The other method for preparing ristocetin-unresponsive platelets was modified from the technique of Olson et al.\(^{16}\) One-day- or two-day-old platelet concentrates from the NIH Clinical Center Blood Bank (prepared in ACD) were centrifuged at 1200 g for 3 min to decrease red blood cell contamination. The platelets were then gel filtered on a 2.5 x 30-cm siliconized glass column packed to a height of 20 cm with Sepharose 2B as described by Tangen and Berman.\(^{17}\) Treatment of the gel, packing the column, gel filtration, eluant flow (1 ml/min), and buffer (without albumin) were as described.\(^{17}\) The gel-filtered platelet (GFP) fractions (3.5 ml per tube) with platelet counts greater than 300,000 were pooled, the volume brought to 50 ml with buffer, and adjusted to 3 mM (Na₂EDTA), and then centrifuged at 2500 g for 10 min. The platelet button was resuspended in buffer to a final concentration of 300,000 platelets per cu mm.

In this assay, the procedure consisted of incubating 0.4 ml of washed GFP with 50 µl of test material for 1 min and then adding 10 µl of a 50 mg/ml solution of ristocetin (final concentration, 1.1 mg/ml). In both assay procedures, the initial slope of aggregation was determined for a pool of normal plasma or a lyophilized reference plasma (Hyland AHF-PTC-control) and various dilutions of these materials from undiluted to 1/16. A standard curve was prepared by plotting the initial slope versus the percentage of plasma in the sample on semilogarithmic graph paper. The standard curve was determined at the beginning and at the end of each group
Table 1. Effect of Normal Cryoprecipitate Column Fractions on Ristocetin Aggregation of vWd Platelets, Washed Normal Platelets, and Gel-filtered and Washed Normal Platelets

<table>
<thead>
<tr>
<th>Column Fraction*</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWd Platelets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ristocetin aggregation restoration (%) of normal plasma</td>
<td>175</td>
<td>34</td>
<td>22</td>
<td>8</td>
<td>7</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>Per cent per 0.1 OD280 nm</td>
<td>45.4</td>
<td>9.6</td>
<td>5.0</td>
<td>0.3</td>
<td>0.2</td>
<td>—</td>
</tr>
<tr>
<td>Washed normal platelets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ristocetin aggregation restoration (%) of normal plasma</td>
<td>210</td>
<td>43</td>
<td>15</td>
<td>10</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Per cent per 0.1 OD280 nm</td>
<td>64.9</td>
<td>12.3</td>
<td>3.8</td>
<td>0.3</td>
<td>&lt; 0.2</td>
<td>—</td>
</tr>
<tr>
<td>vWb platelets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ristocetin aggregation restoration (%) of normal plasma</td>
<td>137</td>
<td>48</td>
<td>20</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Per cent per 0.1 OD280 nm</td>
<td>63.7</td>
<td>19.4</td>
<td>1.3</td>
<td>0.2</td>
<td>0.2</td>
<td>—</td>
</tr>
<tr>
<td>Gel-filtered platelets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ristocetin aggregation restoration (%) of normal plasma</td>
<td>145</td>
<td>40</td>
<td>15</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Per cent per 0.1 OD280 nm</td>
<td>67.0</td>
<td>16.0</td>
<td>1.0</td>
<td>&lt; 0.2</td>
<td>&lt; 0.2</td>
<td>—</td>
</tr>
</tbody>
</table>

*Fraction 1, 48–61 ml; fraction 2, 62–75 ml; fraction 3, 76–89 ml; fraction 4, 90–103 ml; fraction 5, 104–116 ml; the V0 is 48 ml.
†vWd is a woman with 12% factor VIII, a > 30-min bleeding time, abnormal platelet retention, and absent ristocetin aggregation.
‡vWb is a woman with 15% factor VIII, a > 20-min bleeding time, abnormal platelet retention, and absent ristocetin aggregation.
OD, optical density.

of assays, and results were averaged. It was observed that the washed platelets spontaneously regained their aggregation response to ristocetin after standing at room temperature for several hours. Periodic controls (washed platelets and ristocetin) were run throughout the assay procedure, and the test series was terminated if aggregation was observed. The initial slopes produced by the unknown test materials were converted into a percentage of vWF by reference to the standard curve of normal plasma. All test materials were diluted so that they fell near the middle of the standard curve. The sensitivity of the assay varied between less than 3% and less than 6% of normal plasma. No sample was assigned a value below the lower limits of the assay sensitivity. vWF activity was expressed as per cent of normal plasma and specific vWF activity as per cent of normal plasma per 0.1 OD280 (Tables 1 and 2).

RESULTS

Gel Filtration and Polyacrylamide-gel Electrophoresis

The recovery of procoagulant factor VIII in the normal cryoprecipitate varied between 41% and 69%, with a mean of 55.5%, in 13 individuals. After digestion and chromatography of the cryoprecipitate on Sepharose 4B, the recovery of procoagulant factor VIII varied between 50% and 63%, with a mean of 56.2%. After alpha-chymotrypsin digestion of cryoprecipitate, procoagulant factor VIII levels were within ±11% of the predigestion level.

The f.VIII/vWF protein and procoagulant elution patterns of normal and hemophilic-digested cryoprecipitate from a Sepharose 4B column have previously been described. In brief, the procoagulant factor VIII activity
Table 2. Restoration of Ristocetin-induced Aggregation of Normal Washed or Gel-filtered and Washed Platelets by Column Fractions

<table>
<thead>
<tr>
<th>Source</th>
<th>Column Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal* (7)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>53.2 ± 15.6</td>
</tr>
<tr>
<td></td>
<td>bI</td>
</tr>
<tr>
<td>Hemophilia I</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>b</td>
</tr>
<tr>
<td>Hemophilia II</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>b</td>
</tr>
<tr>
<td>vWd I a</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>b</td>
</tr>
<tr>
<td>vWd II a</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>b</td>
</tr>
<tr>
<td>vWd III a</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>b</td>
</tr>
</tbody>
</table>

*All values are expressed as the percentage of normal plasma. For all values greater than 100% of normal plasma, the sample was diluted until the slope could be read from the standard curve.

†These values are the percentage of normal plasma per 0.1 OD of the test sample at 280 nm. Mean ± SD for seven normals.

‡These values are the percentage of normal plasma for each fraction: fraction 1, 48-61 ml; fraction 2, 62-75 ml; fraction 3, 76-89 ml; fraction 4, 90-103 ml; fraction 5, 104-117 ml. Mean of seven normals.

§Patients are described in the text.

in normals appeared at the V0 (48 ml) and eluted primarily in the first 25-30 ml, although small amounts of procoagulant activity eluted later (Fig. 1). A small protein peak (OD280 = 0.169 ± 0.058, mean ± 1 SD; n = 13; observed range, 0.089-0.250) was associated with this procoagulant activity. The V0 from hemophilic cryoprecipitates also had a small protein peak (OD280 = 0.109 mean; range, 0.106-0.120). No discernible protein peak was seen at the V0 in the chromatographed vWd cryoprecipitates (OD280 = 0.020 mean; range, 0.014-0.030). Procoagulant activity was not detected in any portion of the hemophilic or vWd column eluates (Fig. 2), while in the normals the peak factor VIII procoagulant activity clotted in 55.3 ± 13.3 sec (mean ± 1 SD; n = 13; observed range, 33.9-73.3 sec or 10%-41% of pooled normal plasma).

The gel filtration of the normal plasma was almost identical to normal cryoprecipitate in regard to the elution pattern of procoagulant factor VIII. Although the levels were reduced, the protein elution patterns were identical to those found with cryoprecipitate. Chromatography of plasma from patients vW I, II, and III revealed no protein, procoagulant activity, or antigen in any column fraction.

The column eluate was pooled into five fractions (see Materials and Methods). Electrophoresis of the normal cryoprecipitate column fractions in SDS-5% polyacrylamide gels revealed in fractions 1 and 2 a protein that did not enter the gel, while fractions 3, 4, and 5 contained the remainder of the eluted protein without any protein at the top of the gel. When fractions 1 and 2 were reduced and then subjected to SDS-5% polyacrylamide-gel electrophoresis, a major band was present with an estimated molecular weight of 235,000 (Fig. 3).
Fig. 1. The chromatogram of normal cryoprecipitate on Sepharose 4B at 4°C. Eleven to twelve milliliters of cryoprecipitate were digested with alpha-chymotrypsin at 24°C, and the digested cryoprecipitate was gel filtered. The dashed line represents the factor VIII activity and the solid line the absorbance at 280 nm. The numbers and the bars represent the mean and 1 SD for 13 separate experiments with normal cryoprecipitate. The arrow indicates the V₈ (48 ml). The brackets at the top of the figure represent the five column fraction pools (see Materials and Methods).

Fig. 2. The chromatogram of the vWd cryoprecipitate on Sepharose 4B at 4°C. The dashed line represents factor VIII activity and the solid line absorbance at 280 nm. The arrow indicates the V₈ (48 ml), and the brackets indicate the five column fraction pools (see Materials and Methods).
This band could not be identified in any other column fraction. The column fractions of hemophilic cryoprecipitate, reduced and unreduced, behaved identically in polyacrylamide-gel electrophoresis (Fig. 4). Fractions 1 and 2 of the vWd-digested cryoprecipitates did not demonstrate any protein at the top of the SDS–5% polyacrylamide gels, and, after reduction, no subunit of any size was observed (Fig. 5). Fractions 3, 4, and 5 from the vWd cryoprecipitates were similar to the normal and hemophilic fractions.

The 235,000-dalton subunit band from the reduced V₀ fractions (fractions 1 and 2) of normal and hemophilic cryoprecipitates was stained by the PAS reagent, whereas this reagent did not stain any of the gels from the V₀ fractions of the three patients with vWd.

Quantitation of von Willebrand Factor Activity

In initial experiments, column fractions were added to the PRP of vWd patients whose platelets did not aggregate in response to ristocetin. The column
Fig. 4. The SDS-polyacrylamide-gel electrophoresis of the reduced protein fractions (1–5, see Materials and Methods) from chromatographed hemophilic cryoprecipitate. Treatment of samples for electrophoresis is described in Materials and Methods.

fractions from normals and from hemophiliacs with the large-molecular-weight glycoprotein (and the 235,000-dalton subunit) corrected the ristocetin aggregation of the vWd PRP (Table 1).

Identical normal cryoprecipitate column fractions were tested for their ability to induce ristocetin aggregation of vWd patients and normal washed or gel-filtered platelets. The same column fractions (fraction 1) completely corrected the ristocetin aggregation in both test systems (Table 1). This was true whether calculated as total activity or specific activity (activity per 0.1 OD U). Fraction 2 partially corrected the ristocetin-induced platelet aggregation. The
normal and hemophilic samples restored ristocetin aggregation, with fractions 1 and 2 being the most potent. The other column fractions of normal and hemophilic cryoprecipitates had reduced amounts of activity. One freeze-thaw treatment of these samples had less than a 10% effect on the vWF activity compared to the fresh sample. By contrast, the vWd cryoprecipitate fractions were ineffective in restoring ristocetin-induced platelet aggregation (Table 2). The cryoprecipitate supernatant of normal, hemophilia A, and vWd did not have any vWF activity.

The ristocetin-induced aggregation of normal washed or gel-filtered platelets was corrected with normal or hemophilic V₀ fractions (80%–180% and 62%–
70% of the lyophilized standard plasma, respectively; Tables 1 and 2). Fifty normal plasmas from blood bank donors had a mean value of 88% ± 18% compared to the same lyophilized standard plasma (mean ± SD). In all the ristocetin experiments, the amount of Vn normal or hemophilic protein necessary to correct the ristocetin-induced platelet aggregation ranged from 22 to 32 μg/ml final concentration (Tables 1 and 2).

Quantitation of Antigen

The specific antigenic activity (highest reactive dilution per 0.1 OD280) of normal cryoprecipitate was at a maximum in the first fraction (31.8 ± 7.6, mean ± SD; observed range, 22.8–41.6). The second fraction had a mean of 8.0 ± 2.6 antigen units, with a range of 5.5–11.4 (Table 3). Fractions 3, 4, and 5 had correspondingly reduced amounts of antigen in each fraction, and in no instance did any of these fractions have more specific antigenic activity or a higher reactive dilution than fractions 1 and 2. The ratio of antigenic activity of fraction 1 to fraction 2 was 3.5, with a range of 3.1–4.1. In the two patients with hemophilia, the specific antigenic activity fell within the normal range and had a similar fraction 1 to fraction 2 ratio. The three patients with vWD all had reduced or absent levels of antigen in fraction 1 (Table 3). In two of these patients, vW I and vW II, no antigen was detected in any of the column fractions. In patient vW III, a small amount of antigen was observed in fraction 1. The other column fractions had no detectable antigen. Antigen was undetectable in the supernatant from the cryoprecipitate of the three vWD patients.

Correlation of von Willebrand Factor, Procoagulant, and Antigenic Activity

In normals, the peak of specific antigenic activity, specific vWF activity, and specific procoagulant activity appeared consistently in fraction 1, with fraction 2 having less activity but more than that of the other fractions. The ratio of specific vWF activity to specific antigenic activity was 1.43 (observed range, 1.00–1.74) in fraction 1, and 0.91 in fraction 2 (observed range, 0.71–1.10). The mean total f.VIII units per 1.0 OD280 in fractions 1 and 2 were 3.11 and 1.08. The ratio of specific f.VIII to specific antigen units in fractions 1 and 2 of normal were 9.8 and 14.0, respectively. In hemophilias I and II, the ratio of
specific vWF activity to specific antigen activity was 0.78 and 0.65 in fraction 1, and 0.40 and 0.39 in fraction 2, respectively. In the column fractions from vW I and vW II, there was neither antigenic activity nor vWF activity. In vW III, there was a small amount of antigen and vWF activity in fraction 1; the ratio of this vWF activity to antigenic activity was 2.0.

DISCUSSION

Purified f.VIII/vWF is a macromolecular glycoprotein\(^2-4\) with an estimated molecular weight in excess of 2 million daltons. This protein does not enter 3% or 5% SDS-polyacrylamide gels even after prolonged periods of electrophoresis. After reduction it yields a single major subunit on polyacrylamide-gel electrophoresis. Molecular-weight estimations of this subunit vary from 195,000 to 240,000 daltons.\(^2-5\) When isolated from normal plasma, this protein is associated with procoagulant activity. A protein made up of subunits with a similar molecular weight, but devoid of procoagulant activity, has been purified from the plasma of patients with hemophilia A. The absence of immunologic activity in vWd plasma has been used to indicate that this protein is not present. However, attempts to isolate this or other proteins from vWd materials have not been reported.

By the technique of alpha-chymotrypsin digestion of normal or hemophilic cryoprecipitates, we obtained V\(_0\) fractions that have a single major protein band and a single subunit by polyacrylamide-gel electrophoresis. Normal fractions have both procoagulant and vWF activity, thereby suggesting that this protein expresses both of these properties. In hemophilic fractions, only vWF activity and not procoagulant activity is associated with this protein. Void volume fractions (fractions 1 and 2) from three patients with severe vWd were different from normal fractions in four properties: (1) they were devoid of the high-molecular-weight protein or its 235,000-daltons subunit, (2) they did not restore the ristocetin-induced aggregation of plasma-free normal platelets or vWd platelets, (3) they were devoid of procoagulant activity, and (4) they were minimally reactive or unreactive in immunoprecipitation reactions with a monospecific goat or rabbit anti-f.VIII/vWF antibody.\(^19\)

Bouma et al.\(^10\) corrected vWd platelet retention with V\(_0\) fractions from normal and hemophilic cryoprecipitates. Weiss and Rogers\(^9\) demonstrated that normal and hemophilic cryoprecipitates increased platelet retention in patients with vWd. Weiss et al.\(^20\) later showed that the protein which corrected platelet retention was similar to anti-hemophilic factor (AHF), since the column fractions of normal cryoprecipitate which corrected the vWd platelet retention defect were associated with procoagulant activity.

Howard and Firkin\(^21\) first described the use of ristocetin as a platelet aggregating agent and found that the PRP of some patients with vWd had a reduced or absent response. Meyer et al.\(^22\) quantitated the dose response of normal plasma required to correct the ristocetin aggregation of vWd PRP. Recently Weiss et al.\(^11,23\) demonstrated that (1) the plasma factor necessary for ristocetin-induced platelet aggregation was deficient in vWd and (2) this defect was corrected by the V\(_0\) fractions of normal and hemophilic cryoprecipitates, but not by vWd cryoprecipitate. The above studies did not attempt further isolation or
biochemical characterization of the particular protein(s) in the V₀ fractions responsible for the restoration of these functions. Any protein or aggregation of proteins with an effective molecular weight greater than the exclusion limits of the gel would have been included in these V₀ fractions.

Several recent reports suggested that vWF and procoagulant activities of cryoprecipitate or other crudely purified material could be dissociated by high salt concentrations. However, the molecular nature of the procoagulant material has thus far defied characterization. In addition, multiple attempts to repeat this dissociating process with more purified preparations have been unsuccessful. These features have made complete acceptance of a bimolecular model premature.

Our data on normal cryoprecipitate fractions suggest functional heterogeneity of the f.VIII/vWF protein. The specific vWF, antigenic, and procoagulant activities are consistently greater in fraction 1 than fraction 2. This finding suggests that the f.VIII/vWF protein eluting first (highest apparent molecular weight) is more active in these assays per unit of weight than the f.VIII/vWF protein eluting later. Analysis in reference to f.VIII/vWF antigen reveals that, while the vWF per antigen unit is greatest in fraction 1, the procoagulant activity per antigen unit is slightly greater in fraction 2. The hemophilic cryoprecipitates give the same pattern of vWF per antigen unit, although the absolute values are somewhat reduced. As these samples are devoid of procoagulant activity, no analysis of f.VIII per antigen could be made. From these data we tentatively conclude that (1) f.VIII/vWF antigen is not quantitatively equivalent to f.VIII/vWF protein since variations in antigen activity per unit protein are observed in fractions 1 and 2, (2) f.VIII/vWF protein eluting with the largest apparent molecular weight is most active in the functional assays per unit protein, and (3) analysis of vWF and f.VIII procoagulant activity per antigen unit reveals functional heterogeneity, with the later eluting material more effective in a procoagulant f.VIII assay per antigen unit than the earlier eluting material.

In our studies of normal and hemophilia A, the vWF activity was associated with a high-molecular-weight protein apparently composed of repetitive subunits. This protein reacted in immunoprecipitation tests with heterologous monospecific anti-f.VIII/vWF antibodies. The absence of vWF, antigenic, and procoagulant activity in vWD was associated with the absence of this protein on gel chromatography and polyacrylamide-gel electrophoresis. As vWF activity is one of the major biologic functions of this protein, the purified normal protein should currently be designated as the f.VIII/vWF protein.

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