Porcine Factor VIII:C Prepared by Affinity Interaction With von Willebrand Factor and Heterologous Antibodies: Sodium Dodecyl Sulfate Polyacrylamide Gel Analysis

By Gaylord J. Knutson and David N. Fass

A procedure was developed to concentrate and partially purify porcine factor VIII:C from plasma by using conventional precipitation and chromatographic techniques. Blood from heparinized animal(s) was collected in citrate, soybean trypsin inhibitor, ε-aminocaproic acid and benzamidine. After aluminum hydroxide adsorption, polyethylene glycol-6000 precipitation, QAE-cellulose chromatography, and dextran sulfate-agarose chromatography, the factor VIII:C concentrate was 8,100-fold purified with an overall yield of 24%, and thrombin treatment of the factor VIII:C gave an activation coefficient of up to 56. The activation coefficient of plasma factor VIII:C averaged 35. Affinity chromatography of the factor VIII:C concentrate on von Willebrand factor-agarose produced a two fold increase in specific activity. This product was applied to a second affinity resin, the acidic IgG fraction of human antihuman factor VIII:C coupled to agarose. The inactive material which eluted at pH 2.8 from this column and from a similarly prepared nonimmune IgG-agarose column was analyzed by SDS–PAGE. The material uniquely eluted from the immune agarose was represented by protein bands with apparent molecular weights of 166,000, 130,000 and 76,000 and also retained some remnant antigen activity in antibody neutralization studies. Thrombin activated factor VIII:C (40 fold) from von Willebrand factor-agarose chromatography was also specifically bound only by the antifactor VIII:C-agarose. The inactive material which eluted from the antibody column contained polypeptides with apparent molecular weights of 76,000, 67,000 and 50,000. We have concluded that the material purified by two different affinity reagents and visualized by the SDS–PAGE gel represents at least, in part, polypeptides derived from porcine factor VIII:C.

Porcine factor VIII:C and von Willebrand factor form a noncovalent molecular complex in plasma. Porcine factor VIII:C is analogous to that protein which is absent or abnormal in classic human hemophilia, and von Willebrand factor is that protein which is absent or abnormal in human and porcine von Willebrand’s disease. The von Willebrand factor has been purified and partially characterized from various plasma sources, human, bovine, canine, and porcine. However, the purified factor VIII:C protein has not been extensively studied.

Bovine factor VIII:C was purified in sufficient quantity to study by sodium dodecyl salt–polyacrylamide gel electrophoresis (SDS–PAGE). Vehar and Davie reported that their highly purified preparation of bovine factor VIII:C migrated as a triplet on SDS/urea–PAGE with apparent molecular weights of 93,000, 88,000, and 85,000. When treated with thrombin to obtain maximum factor VIII:C activity, the triplet was converted to a doublet with molecular weights of 73,000 and 69,000, a faint, diffuse staining band of about 55,000 molecular weight, and a band of 38,000 molecular weight.

A number of investigations have shown that the factor VIII:C activity can be dissociated from the von Willebrand factor in the presence of 1 M sodium chloride or 0.25 M calcium chloride. This phenomenon has been demonstrated for several species and by several techniques: human, bovine, and canine by gel filtration, porcine by sucrose density gradient centrifugation, and human by immunoadsorption of the von Willebrand factor/factor VIII:C complex by antibodies to von Willebrand factor.

Immunological assays have been developed to measure the factor VIII:C antigen in various plasmas and sera. These assays are based upon the immunoglobulins which arise either spontaneously or during treatment of a classical severe hemophiliac. The antigenic determinants related to the factor VIII clotting activity appear to be measured by the assays. Using SDS–PAGE analysis, Weinstein has attempted to visualize the protein(s) to which these antibodies are binding. The immune complexes formed by the addition of [125I]–labeled (antihuman factor VIII)–Fab to whole human plasma were detected by autoradiography after SDS–PAGE. The resulting autoradiographs showed several bands, one of which was thrombin sensitive.

In this report we have attempted to demonstrate which protein(s) can be attributed to porcine factor VIII:C activity. To carry out this study we have utilized the observation that factor VIII:C and von Willebrand factor form a noncovalent, easily dissociated complex. Additionally, we have utilized the specific affinity of a cross reacting antibody, the...
antibody which developed in a hemophiliac in response to transfusions. The protein removed from solution by these two affinity reagents has been visualized by SDS–PAGE.

MATERIALS AND METHODS

Normal porcine blood was obtained from animals maintained at the Institute Hills Farm, Mayo Foundation, Dr. Paul E. Zollman, Director. The factor VIII:C–deficient plasma was purchased from George King Biomedical, Overland Park, KS. The APTT reagent was a product of General Diagnostics, Morris Plains, N.J. QAE–cellulose was purchased from Schleicher and Schuell, Inc., Keene, NH, and prepared by the method of Sophianopolous and Vestling.23 The DE–52 was a product of Whatman, Inc., Clifton, NJ. The Bio–Gel A–15m (4% agarose) was purchased from Bio–Rad Laboratories, Richmond, CA. The acrylamide and N,N′–methylenebisacrylamide were products from Eastman Chemical Company, Rochester, NY, and were recrystallized from chloroform and 1–propanol, respectively. The proteins for the molecular weight standard were from Dr. J. Reynolds (myosin) and Sigma Chemical Company (β–galactosidase, bovine serum albumin, catalase, ovalbumin). Bovine α–thrombin purified as described26 was a generous gift of Dr. K. G. Mann, Hematology Research, Mayo Clinic, and had a specific activity of 2,700 NIH units/mg. The hemophilic plasma containing the antifactor VIII:C antibody was a gift of Dr. E. J. W. Bowie, Hematology Research, Mayo Clinic. Dansylarginine N–(3–ethyl–1,5–pentanediyl)amide (DAPA) was a gift from Drs. M. E. Nesheim and K. G. Mann, Hematology Research, Mayo Clinic. All other reagents were of reagent grade and were obtained from J. T. Baker Chemical Company, Sigma Chemical Company, Aldrich Chemical Company, and Pierce Chemical Company.

Blood Collection

Blood was collected by femoral veni- or arterio–puncture from normal animals into 1/10 volume of anticoagulant containing 3.8% sodium citrate, 0.5 M e–aminocaproic acid, 0.1 M benzamidine and 50 µg/ml soybean trypsin inhibitor. Five minutes prior to the blood collection, the animals were given an intravenous injection of heparin, 55 units/kg. The blood was collected from either one or two animals.

Preparation of Adsorbed Plasma

The anticoagulated blood was centrifuged at 6000 × g for 10 min at 20°C to remove cells. The plasma was adsorbed with a 1/10 volume of Al(OH)3 (70 mg/ml solids) at 20°C for 10 min. The Al(OH)3 was removed by centrifugation at 6000 × g for 10 min at 20°C. The prothrombin time of the adsorbed plasma was greater than 300 seconds (control 50–60 sec).

Polyethylene Glycol (PEG) Precipitation

A precipitate was formed by adding a sufficient volume of 50% (w/v) solution of PEG–6000 to the adsorbed plasma so that the final concentration of PEG was 5%. The addition was made dropwise with stirring at 20°C, and the resulting suspension was stirred for 20–30 min at 20°C. The precipitate was collected by centrifugation at 6000 × g for 10 min at 20°C.

QAE–Cellulose Chromatography

The PEG precipitate was dissolved in 10 mM histidine, 5 mM CaCl2, 1 mM benzamidine, 0.15 M NaCl, pH 6.0 buffer to 1/5 plasma volume at 20°C. The solution was clarified by centrifugation at 6000 × g for 10 min at 20°C. The supernatant was applied to a bed (8.5 cm diameter × 2.5 cm) of QAE–cellulose resin packed into a coarse sintered glass funnel. After the sample was applied, the resin was washed with 2–3 sample volumes of buffer until the A280 of the effluent was less than 0.50. The proteins were eluted with 10 mM histidine, 5 mM CaCl2, 1 mM benzamidine, 0.80 M NaCl, pH 6.0 buffer. The first 70–80 ml of eluate were discarded and the next 150 ml collected. This 150 ml fraction contained the bulk of the factor VIII:C activity and was designated as QAE–factor VIII:C. The chromatography was carried out at 20°C and was completed in 15–20 min.

Dextran Sulfate–Agarose Chromatography

Dextran sulfate–agarose was prepared according to the method as described by Kisiel.25 The chromatography was performed on a 2.4 × 4.5 cm column equilibrated with 10 mM histidine, 5 mM CaCl2, 1 mM benzamidine, 0.1 M NaCl, pH 6.0 buffer. One half of the QAE–factor VIII:C was processed at a time. Seventy five ml of QAE–factor VIII:C was diluted to 100 ml with column buffer, and applied to the dextran sulfate–agarose at a flow rate of 60–80 ml/hr. The resin was washed until the A280 was less than 0.01, and the proteins were eluted with 10 mM histidine, 1 mM benzamidine, 0.1 M NaCl, 0.255 M CaCl2, pH 6.0 buffer at a flow rate of 20–25 ml/hr and at 20°C.

von Willebrand Factor–Agarose Chromatography

An aliquot (50–100 units) of the eluate from the dextran sulfate–agarose was dialyzed against 10 mM histidine, 5 mM CaCl2, 1 mM benzamidine, 0.1 M NaCl, pH 6.0 buffer to lower the CaCl2 concentration to less than 10 mM. The dialyzed sample was applied to a 1.6 × 2.0 cm column of von Willebrand factor–agarose at a flow rate of 15–20 ml/hr and at 20°C. The resin was washed until the A280 was less than 0.01 and the proteins were eluted with 10 mM histidine, 1 mM benzamidine, 0.10 M NaCl, 0.255 M CaCl2, pH 6.0 buffer at a flow rate of 15–20 ml/hr.

Antifactor VIII:C–Agarose Chromatography

Aliquots of the eluate from the von Willebrand factor–agarose were applied to a 0.9 × 1.6 cm column of antifactor VIII:C–agarose at a flow rate of 15–20 ml/hr. The resin was washed with 10 mM histidine, 5 mM CaCl2, 1 mM benzamidine, 0.5 M NaCl, pH 6.0 buffer prior to elution with 0.1 M glycine, 0.5 M NaCl, pH 2.8. An analogous column of nonimmune immunoglobulin–agarose was used as a control resin. This chromatography was carried out at 20°C.

SDS–PAGE

Analytical SDS–PAGE was performed according to the method of Weber and Osborn with some modification.24 The gel buffer was 0.1 M H3PO4 titrated to pH 6.8 with solid Tris base and the sample preparation buffer contained 6 M urea. The concentration of acrylamide was 5% and electrophoresis was carried out at 5 ma/gel. The standard proteins were myosin (210,000), E. coli β–galactosidase (116,000), bovine serum albumin (69,000), catalase (60,000), and ovalbumin (43,000). The gels were stained with Coomassie Brilliant Blue R–250.

Factor VIII:C Assay

Factor VIII:C was assayed by the one stage activated partial thromboplastin time assay.25 The standard assay procedure was as follows: 10 µl sample, 90 µl factor VIII:C deficient plasma, and 100 µl of APTT reagent were incubated at 37°C for 5 minutes. The assay was initiated by the addition of 100 µl of 20 mM CaCl2. Because some of the fractions contained CaCl2, the assay was modified as follows: 90 µl factor VIII:C deficient plasma and 100 µl of APTT
The von Willebrand factor-agarose was prepared by coupling purified porcine von Willebrand factor to carbodiimide activated denatured bovine serum albumin-agarose (BSA-agarose). The denatured BSA-agarose was prepared by the method described by Parikh, et al. Bovine serum albumin in 10 M urea, 0.2 M NaHCO3, pH 9.0, was coupled to cyanoagen bromide (CNBr) activated27 4% agarose at 1 mg/ml of resin. Five ml of the denatured BSA-agarose was activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl at 0.1 M in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.0 for 30 min at 20°C. The resin was quickly washed with cold buffer (less than 60 sec), and the moist resin cake was added to 25 ml 0.1 M MES, pH 6.0 buffer containing 100 units of purified porcine von Willebrand factor, as assayed by the ristocetin induced platelet aggregation assay.28 The coupling was allowed to proceed overnight at 4°C.

Preparation of Affinity Resins: von Willebrand Factor-Agarose

Antifactor VIII:C antibodies were isolated from the plasma of a 49 yr old multitransfused patient with severe classical hemophilia. This plasma contained 800 Bethesda Inhibitory Units/ml or 53 Bethesda Inhibitory units/mg IgG in the standard Bethesda assay.29 When porcine plasma was used as the source of factor VIII:C, the inhibitory plasma contained 145 Bethesda Inhibitory units/mg IgG. The IgG concentration was determined by Mancinni radial immunodiffusion in a Tripartigen assay (Calbiochem–Behring Corporation, LaJolla, CA.) using standards provided by the manufacturer. The IgG was isolated as follows: 20 ml of plasma was recalcified with 1/10 volume of 0.2 M CaCl2 and bovine α-thrombin was added to a final concentration of 5 units/ml. After incubation at 37°C for 30 min, the fibrin clot was removed. Saturated ammonium sulphate was added to a final concentration of 35% and the pH was adjusted to 8.0 with 3 M NaOH. After stirring for 30 min at 20°C, the precipitate was collected by centrifugation and dissolved in 0.04 M phosphate, pH 8.0 buffer (0.04 M Na2HPO4, titrated with 0.04 M KH2PO4) to original plasma volume and dialyzed overnight. The protein was applied to a 2.5 x 15 cm column of DEAE-cellulose (DE-52) equilibrated with 0.04 M phosphate, pH 8.0. The column was washed until the A280 was less than 0.10 and the adsorbed proteins were eluted with 0.4 M phosphate, pH 7.0 buffer. The eluted proteins were dialyzed against 0.05 M imidazole, 0.1 M NaCl, pH 7.0. The immunoglobulins in this fraction were isolated on a 1.5 x 2.5 cm column of protein A-Sepharose. The immunoglobulins were eluted with 0.1 M glycine, 0.5 M NaCl pH 3.0 buffer into 1/10 volume of 1 M Tris pH 8.5. These acidic IgG antifactor VIII:C antibodies contained antifactor VIII:C activity at 1200 Bethesda Inhibitory units/ml or 460 Bethesda Inhibitory units/mg IgG using human plasma and 162 Bethesda Inhibitory units/ml or 62 Bethesda Inhibitory units/mg IgG using porcine plasma. This acidic IgG fraction contained 45% of the inhibitory activity and 5% of the IgG. These antibodies were coupled to CNBr activated agarose as described by March, et al. at 1 mg/ml of resin. An analogous fraction of IgG was prepared from normal plasma and subsequently coupled to agarose by the same procedure.

Preparation of the Factor VIII:C Concentrate

The preparation of the factor VIII:C concentrate for the two step affinity analysis included measures to prevent possible proteolysis, particularly by thrombin. Thus, the blood was collected from a heparinized animal(s) by femoral veni- or arterio-puncture into an anticoagulant containing the protease inhibitors, benzamidine, soybean trypsin inhibitor and e-aminocaproic acid. After removing the cells, plasma was adsorbed with Al(OH)3, an effective adsorbent of the vitamin K-depent factors.

The first two steps of the procedure were precipitation of proteins in the adsorbed plasma with PEG–6000 and subsequent batchwise chromatography of the precipitated proteins on QAE–cellulose. These steps yielded a product which was 200 to 300 fold purified, which had the same activation coefficient as the factor VIII:C in plasma, and which represented a 40 to 50% yield. The QAE–cellulose product remained stable at 4°C for a week, but routinely the next step was carried out immediately.

The last step in the preparation was chromatography on dextran sulfate-agarose (Fig. 1). In addition to an increase in specific activity, this step separated the factor VIII:C from the bulk of the von Willebrand
factor. This factor VIII:C concentrate had no ristocetin induced platelet aggregation (RIPA) activity but porcine von Willebrand antigen was detectable by the immunoradiometric assay (IRMA) activity but porcine von Willebrand factor (RIPA) activity but was eluted from the resin at a flow rate of 60–80 ml/hr (4 ml/fraction). The resin was washed until the A280 was less than 0.01, and the proteins were eluted with 10 mM Histidine, 1 mM Benzamidine, 0.1 M NaCl, 0.255 M CaCl2, pH 6.0 at a flow rate of 20–25 ml/hr (2 ml/fraction). The chromatography was carried out at 20°C. Protein was measured as A280 (O—O) and factor VIII:C activity was expressed as VIII:C U/ml (●—●) in the left panel or as VIII:C C U/ml (O—O) in the right panel. This chromatography was repeated for the remaining 75 ml of the QAE-factor VIII:C; the final result of this chromatographic step is summarized in Table 1.

The total time for this procedure, blood collection through elution from dextran sulfate–agarose, was 12 to 14 hr. The overall yield was 20–25% and the product retained its activatability by thrombin. Thus, the product was considered suitable for further study. The procedure is summarized in Table 1.

### von Willebrand Factor-Agarose Chromatography

The first affinity step for factor VIII:C utilized the noncovalent reversible association between porcine factor VIII:C and von Willebrand factor. Purified porcine von Willebrand factor coupled to denatured BSA–agarose served as the affinity reagent. In the presence of a low concentration of CaCl2 factor VIII:C activity was efficiently removed from solution (92–99% of the applied) (Fig. 2), and there was a two-fold increase in the specific activity of the factor VIII:C (Table 2). When a sample of von Willebrand factor–agarose factor VIII:C was assayed by the IRMA for von Willebrand antigen, the concentration of von Willebrand factor antigen was found to be 8 x 10^{-14} units/ml (approximately 4 x 10^{-9} gm/ml). When von Willebrand factor was coupled to aminohexyl–Sepharose (AH–Sepharose) by carbodiimide coupling, the resulting resin was also an effective affinity resin for factor VIII:C. Both affinity resins were reusable multiple times, but after 15 to 20 times the eluate contained additional proteins as detected on SDS–polyacryl-

### Table 1. Summary of the Preparation of the Porcine Factor VIII:C Concentrate

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Total Protein</th>
<th>Total Activity VIII:C Units</th>
<th>Activation Coefficient</th>
<th>Specific Activity VIII:C Units/A280</th>
<th>Yield %</th>
<th>Purification (x-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1,000</td>
<td>56,000</td>
<td>52,000</td>
<td>35</td>
<td>0.93</td>
<td>100</td>
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</tr>
<tr>
<td>AI (OH)3 supernatant</td>
<td>1,000</td>
<td>55,900</td>
<td>51,000</td>
<td>30</td>
<td>0.91</td>
<td>98</td>
<td>0.98</td>
</tr>
<tr>
<td>5% PEG-6000 precipitate</td>
<td>200</td>
<td>8,900</td>
<td>42,000</td>
<td>28</td>
<td>4.7</td>
<td>80</td>
<td>5.05</td>
</tr>
<tr>
<td>QAE-cellulose</td>
<td>150</td>
<td>91</td>
<td>18,600</td>
<td>32</td>
<td>204</td>
<td>36</td>
<td>219</td>
</tr>
<tr>
<td>Dextran sulfate–agarose*</td>
<td>32</td>
<td>1.57</td>
<td>11,854</td>
<td>56</td>
<td>7,550</td>
<td>24</td>
<td>8,118</td>
</tr>
</tbody>
</table>

*The summary for this step represents the result of chromatographing the QAE-factor VIII:C in two batches as described in Figure 1.

![Graph 1](image1.png)  
**Fig. 1.** Dextran sulfate–agarose chromatography of factor VIII:C. A 2.4 x 4.5 cm column of dextran sulfate–agarose was equilibrated with 10 mM Histidine, 5 mM CaCl2, 1 mM Benzamidine, 0.1 M NaCl, pH 6.0 buffer. Seventy-five ml of the eluate from QAE-cellulose was diluted to 100 ml with column buffer and applied to the resin at a flow rate of 60–80 ml/hr (4 ml/fraction). The resin was washed until the A280 was less than 0.01, and the proteins were eluted with 10 mM Histidine, 1 mM Benzamidine, 0.1 M NaCl, 0.255 M CaCl2, pH 6.0 at a flow rate of 20–25 ml/hr (2 ml/fraction). The chromatography was carried out at 20°C. Protein was measured as A280 (O—O) and factor VIII:C activity was expressed as VIII:C U/ml (●—●) in the left panel or as VIII:C C U/ml (O—O) in the right panel. This chromatography was repeated for the remaining 75 ml of the QAE-factor VIII:C; the final result of this chromatographic step is summarized in Table 1.

![Graph 2](image2.png)  
**Fig. 2.** Von Willebrand factor–agarose affinity chromatography of factor VIII:C. A 1.8 x 2.0 cm column of von Willebrand factor–agarose was equilibrated with 10 mM Histidine, 5 mM CaCl2, 1 mM Benzamidine, 0.1 M NaCl, pH 6.0 buffer. One hundred units of the factor VIII:C concentrate (eluate from dextran sulfate–agarose) was applied at a flow rate of 15–20 ml/hr (4 ml/fraction) and at 20°C. The column was washed until the A280 was less than 0.01, and the proteins were eluted with 10 mM Histidine, 1 mM Benzamidine, 0.10 M NaCl, 0.255 M CaCl2, pH 6.0 buffer at a flow rate of 15–20 ml/hr (2 ml/fraction) and at 20°C. Protein was measured as A280 (O—O) and factor VIII:C activity expressed as VIII:C C U/ml (●—●).
amide gels, not seen in the eluate from fresh resin. No attempt was made to regenerate the resin.

Immunoglobulin-agarose Chromatography

The second affinity step utilized an antibody to human factor VIII:C isolated from a hemophilic plasma. One milliliter of the antifactor VIII:C-agarose removed 20 to 40 units of factor VIII:C (900 to 1600 units of factor VIII:C) from solution (Table 3). (This was accomplished in a single passage over the column with a total exposure of 15 min.) No factor VIII:C activity was washed from the column with 0.5 M NaCl prior to the column being eluted with 0.1 M glycine, 0.5 M NaCl, pH 2.8 buffer. Because of its sensitivity to low pH, factor VIII:C activity was not detected in the eluate even when the sample was eluted into 1 M Tris, pH 8.5 to neutralize the pH. When an identical solution of factor VIII:C was applied to 1 milliliter of the control resin, nonimmune human IgG coupled to agarose and the column washed with 0.5 M NaCl, 80 to 85% of the activity was not bound by the resin (Table 3). This resin was also eluted with the pH 2.8 glycine buffer. Both of these columns were reusable after 10 cycles of pH changes.

Antibody Neutralization

Thirty units of factor VIII:C (900 units factor VIII:C) were applied to 1 ml of the antifactor VIII:C-agarose. The column was washed and eluted at pH 2.8 into 1/10 volume of imidazole buffered saline containing 1% bovine serum albumin and sufficient 1 M Tris, pH 8.5 to bring the final pH to 7.4. A control nonimmune IgG-agarose column, binding in this case less than 1% of input factor VIII:C, was eluted in a similar fashion. These eluates were used to dilute the acidic IgG fraction of the antifactor VIII:C antibodies prior to a Bethesda Inhibitory assay.

Pairs of dilution curves were established on two separate occasions and the data combined in Fig. 3.

The seven dilutions at which data were collected from the immune or nonimmune human IgG column, are represented by two or three assays for factor VIII:C remaining. The lines are drawn through the average value of the factor VIII:C assays. The apparent reduction of the antibody titre toward factor VIII:C was 1.6 fold suggesting that the acid eluate from the immune IgG column contained approximately 4% of the potential antibody neutralizing activity of the input to the column. (The eluate from the nonimmune column produced a curve which was indistinguishable from that obtained by dilution of antibody with immunoglobulin-agarose column buffer. The 50% titration point was 1:177 N = 2 compared to 1:184 for nonimmune IgG N = 3.) The inability of this antibody to reduce factor VIII:C activity to zero is a characteristic of its activity against porcine, but not human, factor VIII:C sources. The inability is also seen with the intact, unfractionated, antiserum as well as the acidic immunoglobulins.

<p>| Table 2. Chromatography of Factor VIII:C on Porcine von Willebrand Factor-Agarose |
|---------------------------------|------------------|------------------|---------------|------------------|---------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Total Protein A_{280}</th>
<th>Total Activity VIII:C Units</th>
<th>Specific Activity VIII:C Units/A_{280}</th>
<th>Activation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliquot of the factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIII:C concentrate</td>
<td>15</td>
<td>0.74</td>
<td>5,520</td>
<td>7,550</td>
<td>56</td>
</tr>
<tr>
<td>Willebrand factor-agarose eluate</td>
<td>6</td>
<td>0.16</td>
<td>2,160</td>
<td>13,500</td>
<td>30</td>
</tr>
</tbody>
</table>

The seven dilutions at which data were collected from the immune or nonimmune human IgG column, are represented by two or three assays for factor VIII:C remaining. The lines are drawn through the average value of the factor VIII:C assays. The apparent reduction of the antibody titre toward factor VIII:C was 1.6 fold suggesting that the acid eluate from the immune IgG column contained approximately 4% of the potential antibody neutralizing activity of the input to the column. (The eluate from the nonimmune column produced a curve which was indistinguishable from that obtained by dilution of antibody with immunoglobulin-agarose column buffer. The 50% titration point was 1:177 N = 2 compared to 1:184 for nonimmune IgG N = 3.) The inability of this antibody to reduce factor VIII:C activity to zero is a characteristic of its activity against porcine, but not human, factor VIII:C sources. The inability is also seen with the intact, unfractionated, antiserum as well as the acidic immunoglobulins.

Fig. 3. Blocking of anti-factor VIII:C activity by the acid eluate from antifactor VIII:C agarose. The pH 2.8 eluate from an antifactor VIII:C-agarose column which removed 30 units of factor VIII:C (900 units of factor VIII:C) was used to dilute anti-factor VIII:C antibodies prior to a Bethesda Inhibitory assay, using porcine plasma as the factor VIII:C source. The 50% titration point is at a 1:117 dilution of the antibody solution (Θ—Θ). The pH 2.8 eluate from the control, non-immune IgG-agarose column, which removed less than 1% of the factor VIII:C activity, was also used to dilute the anti-factor VIII:C antibodies. The 50% titration point for this eluate is at a 1:184 dilution of the antibody solution (Ο—Ο). The x-axis represents the log of the dilution of the anti-factor VIII:C antibodies being titrated. The original antibody solution contained 2.6 mg IgG/ml and 162 Bethesda Inhibitory Units/ml versus porcine plasma.
SDS–PAGE Analysis

The various fractions associated with the immuno-globulin columns were analyzed by 5% SDS–PAGE (Fig. 4). Gels A–C represent the fractions associated with the nonimmune IgG–agarose and gels D–F are the fractions from the antifactor VIII:C–agarose. The material eluted from the antifactor VIII:C–agarose is seen on gel F. All of the material which was applied to the gel entered the gel under nonreducing conditions. The bands have apparent molecular weights of 166,000, 130,000 and 76,000. The analysis of the same fraction from the nonimmune IgG–agarose, gel C, shows no visible bands. The material not binding to the columns is represented by gel A, nonimmune IgG–agarose, and gel D, antifactor VIII:C–agarose. Gels B and E show the material washed from the two columns with 0.5 M NaCl. By comparing all six gels, it appears that the antifactor VIII:C–agarose removed the middle band of a triplet around 76,000 molecular weight and bands with apparent molecular weights of 166,000 and 130,000. The three bands seen on gel F did not show a reduction of apparent molecular weight upon treatment with 0.14 M 2-mercaptoethanol in 1% SDS at 90°C for 15 min. Actually, the band measured at 76,000 appeared to increase to 83,000 daltons upon reduction suggesting intrachain disulfides.

The effect of thrombin on the factor VIII:C eluted from von Willebrand factor-agarose was determined by treating the factor VIII:C with thrombin prior to applying the sample to the antibody column. For this experiment the eluate from the von Willebrand factor-agarose was dialyzed against 10 mM histidine, 5 mM CaCl₂, 1 mM benzamidine, 0.1 M NaCl to lower the CaCl₂ concentration. Twenty units of factor VIII:C activity (10 U/ml) were treated with thrombin (1 U/ml final concentration) for 5 min at 37°C. The reaction was stopped by the addition of DAPA at a final concentration of 5 × 10⁻⁶ M. The reaction mixture was then applied to the antibody column equilibrated with DAPA containing buffer. The column was washed and eluted at pH 2.8 as described above. A control experiment was done with an analogous aliquot of the same factor VIII:C solution without thrombin treatment. In both the experiment and the control 95–99% of the factor VIII:C activity was removed from solution and no activity was measured when the columns were washed with 0.5 M NaCl.

The SDS–PAGE analysis of this experiment (Fig. 5) shows that the 166,000, 130,000 and possibly the 76,000 bands (Gel A) are sensitive to thrombin. Gel B represents the thrombin treated factor VIII:C which bound to and eluted from the antifactor VIII:C-agarose column. The apparent molecular weights are 76,000, 67,000 and an indistinct band at 50,000. Gel T shows the thrombin control.

DISCUSSION

In this report, we have attempted to demonstrate what protein(s) can be attributed to porcine factor VIII coagulant activity. To do this, we have used antibodies which, by their inhibitory activity, are antibodies to factor VIII:C. Even though these antibodies are antihuman factor VIII:C, there was sufficient cross reactivity of this reagent with porcine factor VIII:C to be useful for the study. In addition to this specific affinity, we utilized the noncovalent, reversible association between porcine factor VIII:C and porcine von Willebrand factor as the last step in the preparation of the sample to be analyzed by the immobilized antibody.

Because factor VIII:C is apparently unstable and is present in plasma in low quantity, we have developed a procedure to concentrate and partially purify factor VIII:C from plasma by using conventional precipita-

* A recent study of the activation of human factor VIII:C by thrombin (Hultin, MB and Jesty, J: Blood 57:476–482, 1981) suggests that activated factor VIII:C persists in the presence of 2 × 10⁻⁴ M DAPA. In our assays, the DAPA in the sample was diluted to 4.2 × 10⁻⁹ M prior to the assay and 1.4 × 10⁻⁸ M in the assay. This concentration of DAPA is 5 orders of magnitude less than the K₅₀ for thrombin, and we would expect expression of activity.
Fig. 5. SDS-5% polyacrylamide gel electrophoretic analysis of thrombin treated von Willebrand factor-agarose affinity purified factor VIII:C. The protein on gel A was eluted at pH 2.8 from the antifactor VIII:C-agarose column after 20 units of factor VIII:C (735 units of factor VIII:C) were bound by the resin (analogous sample is shown in Fig. 4, gel F). The same amount of the affinity purified factor VIII:C was treated with thrombin (1 U/ml, 37°C, 5 min) prior to chromatography on the same antibody column. The protein which was subsequently eluted from the resin at pH 2.8 is shown on gel B. Gel S shows the standard proteins and gel T is the thrombin control gel.

tion and chromatographic techniques. One criterion used to define an appropriate preparation was that the factor VIII:C had to maintain its activatability by thrombin. There have been studies which suggest that factor VIII:C may exist in plasma in a precursor form and final factor VIII:C activity is expressed when it is altered by thrombin and/or factor Xa, calcium and phospholipid.

In the preparation of the partially purified factor VIII:C, measures were taken to prevent proteolysis, especially by thrombin. The blood was drawn by blood vessel puncture rather than using slaughter-house blood. The animals were heparinized prior to bleeding and the blood was collected into a mixture of protease inhibitors. Additionally, the first step in the preparation, after removal of cells, was to adsorb the plasma with aluminum hydroxide, an effective adsorbent for vitamin K-dependent proteins.

The partially purified factor VIII:C which eluted from dextran sulfate-agarose did meet the criterion established for the subsequent analysis. The yield was 20–25%, the activation coefficient was similar to that in the starting plasma (30–40 fold), and the purifica-

tion was 8000 to 10,000 fold. It is not known what factor(s) has stabilized the factor VIII:C at this stage; possibly maintaining the product at pH 6.0 and/or 0.25 M CaCl₂. Tuddenham et al. have indicated that disopropyl fluorophosphate (DFP) is necessary for isolation of stable, unactivated human factor VIII:C by immunoadsorbent chromatography. We did not include DFP at any step and we did not obtain two peaks of factor VIII:C activity eluting from dextran sulfate-agarose if the resin was eluted by a linear gradient from 5 mM to 250 mM CaCl₂. The factor VIII:C elutes between 0.17 M and 0.25 M CaCl₂. Possibly, the precautions we have taken are sufficient to prevent significant proteolysis or porcine factor VIII:C is less susceptible to proteolysis than is human factor VIII:C. Alternatively, the protein which we see on SDS–PAGE is proteolyzed and has arisen from a single chain. These cleavages, however, may not be catalyzed by thrombin and may not cause an increase or decrease in activity or activation coefficient.

The von Willebrand factor-agarose served as an affinity step as indicated by an increase in the specific activity. Horowitz et al. described the properties of "low molecular weight factor VIII" prepared by affinity chromatography using "factor VIII-Sepharose." Their material had no detectable factor IX clotting activity, sedimented with an apparent S value of 7.2, had an VIII:C/VIIIIR:Ag ratio ≥150, and was activatable by thrombin 3–4 fold. Their procedure, while effective in producing a purified product for analysis had a low capacity and required long exposure of the factor VIII:C to the resin at ambient temperature. The difference between their results and ours may be explained by the prior purification steps which we used to remove much of the fluid phase von Willebrand factor. This von Willebrand factor in solution would, of course, be a competitive binder of the factor VIII:C. The efficiency of the affinity step, as we have performed it, probably represents the equilibrium between free and complexed VIII:C as described by Koutts et al. and Over et al. Horowitz et al. did not report an SDS–PAGE analysis of the factor VIII:C; but our experience shows factor VIII:C at this stage still contains many contaminating proteins (Fig. 4, Gel D). In the present study the factor VIII:C, affinity purified using von Willebrand factor-agarose, had no detectable (<1%) factor IX clotting activity, had a VIII:C/VIIIIR:Ag ratio ≥15,000 and was activatable by thrombin 30–40 fold. The difference between the VIII:C/VIIIIR:Ag ratio seen in the analysis by Horowitz et al. and that done here may reflect the sensitivity of the antigen assays used and the factor VIII:C concentration being measured and not the state of dissociation achieved, as their number represents a
minimum estimate. An unexplained observation is that the activation coefficient always decreased slightly after the von Willebrand factor-agarose step, even though it was always 30–40 fold, similar to that of plasma.

The definitive step in this study was the removal of factor VIII:C activity, previously affinity purified on von Willebrand factor-agarose, by the antifactor VIII:C antibody used in this study was isolated from a hemophilic plasma. The particular acidic fraction which was used had the highest specific activity in inhibitory units per mg protein. The SDS-PAGE analysis of the antibody column experiment showed that the antifactor VIII:C antibodies bound proteins, which dissociated at pH 2.8, and had apparent molecular weights of 166,000, 130,000, and 76,000. The samples containing these peptide chains were also able to block factor VIII:C inhibition. These bands did not show a reduction of apparent molecular weight upon treatment with 2-mercaptoethanol in the presence of 1% SDS.

Lane et al.36 described experiments using immobilized antibodies to factor VIII:C isolated from a hemophilic patient. When 4.5 units of factor VIII, purified from a commercial concentrate, were applied to their affinity resin, 45% to 81% of the activity was removed from solution. The SDS-PAGE analysis, under reducing conditions, showed that 95% of the protein on the gel was associated with two bands with apparent molecular weights of 62,000 and 35,000. When an analogous sample was applied to a column prepared by coupling rabbit antifactor VIII antibodies to agarose, the eluate showed eight bands, including the 62,000 and the 35,000 molecular weight bands.

Our study differs from the investigation of Lane et al.36 in several aspects. The factor VIII:C concentrate which was prepared in our laboratory included protease inhibitors in the initial steps; the factor VIII:C was affinity purified on von Willebrand factor-agarose prior to the antifactor VIII:C-agarose step; the antifactor VIII:C–agarose removed 20–40 units (30–40 fold activatable) from solution with no less than 85% efficiency; and the material which eluted from the antibody column entered the SDS-polyacrylamide gel under nonreducing conditions. We also cannot record on film the bands generated by Coomassie Blue stained gels containing the equivalent of 4 units of activity. This, however, may reflect the difference between the intrinsic factor VIII:C activities of the proteins from the two species.

The demonstration that thrombin sensitive peptides from von Willebrand factor-agarose bind to specific antifactor VIII:C antibody agarose provides additional evidence that these bands represent the protein having factor VIII:C activity. This experiment was carried out by incubating the factor VIII:C with thrombin prior to application to the antifactor VIII:C-agarose. When this was done, no detectible factor VIII:C activity passed through the antibody column and proteins with apparent molecular weights of 76,000, 67,000, and 50,000 were eluted.

The bovine factor VIII:C prepared by Vehar and Davie was represented by a triplet of stained bands with apparent molecular weights of 93,000, 88,000, 85,000 which resulted in bands of 73,000, 69,000, 55,000, 38,000 after thrombin treatment. The material which bound to and eluted from our antifactor VIII:C-agarose column after thrombin treatment was represented by bands of 76,000, 67,000, and 50,000. Thus, the two different approaches for studying thrombin activated factor VIII:C yielded similar results even though the "unactivated" material was different. Possibly the unactivated material in our study may have been cleaved by limited proteolysis even though the material is 30–40 fold activatable. Factor VIII:C may be very sensitive to the first cleavage and subsequent cleavages will produce full expression of activity. The difference between our unactivated factor VIII:C and Vehar and Davie's cannot be explained at this time.

Vehar and Davie used an SDS-PAGE system that contained 8 M urea in the gel so that their material would enter a 5% polyacrylamide gel. Our electrophoretic system differed in that the sample preparation buffer contained 6 M urea rather than 8 M urea and our gel buffer contained no urea. As seen in Fig. 4, all the material entered the 5% polyacrylamide gel. If we left the urea out of the sample preparation buffer, the results were identical to those with the sample buffer with urea. The necessity for 8 M urea may be explained by the amount of protein applied to the gels. We applied only 15%–20% of the number of factor VIII:C units that Vehar and Davie applied.

Weinstein found that 125I-labeled (antihuman factor VIII)–Fab bound to proteins that principally migrated at 240,000 molecular weight with minor bands at 180,000 and 130,000 molecular weights. When he treated plasma with thrombin, the 240,000 molecular weight band disappeared and a band at 110,000 molecular weight appeared. These experiments suggested that these bands are in total, or in part, the factor VIII:C antigen. Thrombin treatment of plasma is a complicated situation. We have tried to simplify the experiments by analyzing the carefully prepared partially purified factor VIII:C which eluted from von Willebrand factor-agarose.

The results reported here used an acidic IgG fraction isolated from the plasma of a severe classic
hemophilia patient. This IgG fraction was used, after being labeled with $^{125}$I, as a probe in a binding assay to screen for monoclonal antibodies to porcine factor VIII:C. An accompanying manuscript describes the isolation of the monoclonal antibodies and the characterization of the porcine factor VIII:C polypeptides reacting with the monoclonal antibody. We cannot distinguish between the polypeptides bound by the human inhibitor antibodies and those bound by the mouse monoclonal antibody.

We have concluded that the material affinity purified by two different affinity reagents and visualized on the SDS–PAGE gels represents polypeptides derived from the porcine factor VIII:C. There may be other polypeptides associated with porcine factor VIII:C activity but the antifactor VIII:C–agarose coincidentally removed factor VIII:C activity and the polypeptides seen on the SDS–PAGE (compare Fig. 4, gel A with gel D). This suggests that one or more of these proteins are necessary for factor VIII:C activity. The polypeptides eluted from monoclonal antibody columns are sufficient by themselves for factor VIII:C activity and are indistinguishable from those eluted from the inhibitor antibody column Fig. 4, gel F. In addition, the procedure identified polypeptides which were thrombin sensitive over the time and concentration conditions which activated factor VIII:C to factor VIII:C$_A$. The protein also retained about 4% of the potential antibody neutralizing activity and represented about 5%–10% of the stained protein from the 13,500 fold purified active factor VIII:C, indicating a concentration of these proteins in porcine plasma of about 200 ng/ml. These characteristics, in conjunction with the molecular weight considerations above, provide the comparative data which suggest that the material seen in Fig. 4, gel F and Fig. 5, gels A and B are attributable to porcine factor VIII:C.

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Porcine factor VIII:C prepared by affinity interaction with von Willebrand factor and heterologous antibodies: sodium dodecyl sulfate polyacrylamide gel analysis

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