Human Factor VIII: A Calcium-Linked Protein Complex

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The possible role of Ca$^{2+}$ as an essential constituent part of the human factor VIII complex has been investigated by stability studies, metal determinations, and gel filtration experiments. In citrated plasma, the factor VIII coagulant activity (VIII:C) deteriorated during storage in a biphasic manner. Collection of blood in heparin, instead of chelating anticoagulants, or neutralization of citrate by addition of CaCl$_2$ to heparinized citrate phosphate dextrose (CPD) plasma rendered VIII:C noticeably stable. At physiologic levels of ionized calcium, VIII:C was almost completely stable during incubation of plasma for 6 hr at 37°C. The influence of other divalent ions was also studied. Highly purified factor VIII complex was subjected to atomic absorption spectrophotometric analysis and found to contain about 1.0 mole calcium per 220,000 daltons. This intrinsic calcium could be readily removed by EDTA. When heparin plasma and CPD plasma were chromatographed on Sepharose CL-6B at 37°C, all the factor-VIII-related activities eluted together as large protein complexes. In contrast, factor VIII coagulant antigen (VIII:CAg) and factor-VIII-related antigen (VIII:R:Ag) were completely dissociated upon exposure to EDTA. From these observations it is concluded that human factor VIII circulates in normal plasma as a calcium-linked protein complex.

Despite extensive investigations during the last decade, the precise structure and function of the factor VIII complex has not yet been clarified. Several studies have shown that the factor-VIII-related carrier protein (VIII:R) circulates as a series of large multimers with a relative molecular mass of 1-20 x 10$^6$ daltons.$^{1,2}$ On reduction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), one single band is detected. The relative molecular mass of this VIII:R subunit is approximately 200,000 daltons.$^3$ The coagulant activity (VIII:C) resides in another component, which is associated with the carrier protein by noncovalent bonds. Dissociation of VIII:R and VIII:C has been observed under conditions of high ionic strength or calcium concentrations around 0.25 $M$,$^4$ even in the presence of protease inhibitors.$^5$ Although debated,$^7$ this two-component concept has been further supported by several investigators who have used immunoabsorbent chromatography to purify VIII:C.$^{8,9}$ Hoyer and Trabold$^{10}$ have studied VIII:C separated by this technique and reported a relative molecular mass of 285,000 daltons. The coagulant component constitutes only about 1% of the total amount of circulating factor VIII complex.$^{11}$

Understanding the molecular structure of the factor VIII complex, and especially the characteristics of VIII:C, has been hampered by its pronounced lability. Several investigators have reported that factor VIII in normal citrated plasma loses about 50% of its initial coagulant activity during the first 24 hr of storage. In 1955, Spaet and Garner$^{12}$ showed that the storage instability of VIII:C becomes more pronounced with increasing concentrations of the chelating anticoagulants, citrate, and oxalate. Weiss$^{13}$ studied the influence of different cations on the decay of factors V and VIII in plasma and showed that Ca$^{2+}$, as well as other divalent ions, could prevent the rapid loss of activity. The experiments by Stibbe et al.$^{14}$ showed that Ca$^{2+}$ decreased the rate of inactivation when added to resin plasma (decalcified plasma prepared by ion-exchange chromatography). Rock et al.$^{15}$ raised the question as to whether the numerous conflicting reports concerning the biochemical properties of factor VIII are due to the use of chelating anticoagulants in the collection of blood. These authors found that more than 50% of VIII:C had an apparent molecular mass of about 20,000 daltons when heparinized plasma was studied by gel filtration and ultracentrifugation. In contrast with these findings, Bolhuis et al.$^{16}$ reported that all the factor-VIII-related activities eluted with the void volume when heparinized plasma was chromatographed on Sepharose 6B in the presence of Ca$^{2+}$.

This study was undertaken to further evaluate the possible role of Ca$^{2+}$ as an essential component of the human factor VIII complex. Results from stability studies, metal analysis, and gel filtration experiments are included in this report.

MATERIALS AND METHODS

Materials

Sephadex G-25 Superfine, Sepharose CL-6B, and Sephacryl S-500 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Heparsorb (ion-exchange adsorbent intended for removal of heparin) was purchased from General Diagnostics (Warner Lambert Company, Morris Plains, NJ). Virtually metal-free Tris-acetate buffer was prepared by dissolving the ultrapure reagents Tris (Aristar, BDH Chemicals, Parkstone, England), acetic acid (Suprapur, E. Merck, Darmstadt, Germany), and sodium acetate (Suprapur, E. Merck) in distilled water that had been further purified by
passage over a column with mixed bed resin AG501 X8 20–50 mesh (Bio Rad Laboratories, Richmond, VA).

The following protease inhibitors were used: diisopropyl fluorophosphate (DFP) from Serva Feinbiochemica, Heidelberg, Germany; phenyl methyl sulfonylfluoride (PMSF) from Serva Feinbiochemica; Trasylol (aprotinin) from Bayer (Sweden) AB, (Stockholm, Sweden); and α-dansyl-p-guanidino-phenylalanine-piperidide (S 2581) from KabiVitrum AB, (Stockholm, Sweden). All other chemicals used were of reagent grade.

Assay Methods

Factor VIII Coagulant Activity

Factor VIII coagulant activity (VIII:C) was determined in three ways.

(1) The “Lode one-stage assay” was especially developed for samples containing metal salts. One part “Plarum” (artificial substrate plasma) diluted 1:2 with saline and 1 part Cephotest (Nyggaard and Co, Oslo, Norway) were preactivated at 37°C for at least 30 min. This mixture was then usable for 5 h. Two-tenths milliliter of the activated mixture was mixed with 0.1 ml sample or standard dilution and 0.1 ml 0.015 M CaCl₂. Clotting times were recorded by a Lode Coagulometer (Svenska Labex, Helsingborg, Sweden). The artificial reagent “Plarum” was prepared according to Nyman, from platelet-poor human serum, bovine factor V, and human fibrinogen. The content of VIII:C was less than 0.01 IU/ml. “Hemophilia A” plasma (Georg King Bio-medical Inc., Salem, NC) was used for control experiments. Prior to assaying, all plasma samples (in the stability studies) were treated with Heparin according to the manufacturer’s instructions.

(2) The two-stage assay was performed according to Denson, using combined reagent and substrate plasma from Diagnostic Reagents Ltd. (England), according to the manufacturer’s instructions, with the following modifications: Michaelis Veronal buffer pH 7.4, with 1% BSA was used instead of citrated saline for the dilutions. Al(OH)₃ adsorption was omitted as suggested by Barrowcliffe et al., since a nonsubsampling system and high dilutions (1/200, 1/400, and 1/800) were used.

(3) The “Electra one-stage assay,” based on the activated partial thromboplastin time principle, was used to assess the potency of factor VIII preparations and column fractions. One-tenth milliliter “Plarum” diluted 1:2 with saline, 0.1 ml Cephotest, and 0.1 ml sample or standard dilution were preincubated for 10.5 mm at 37°C in an Electra 600 Automatic clotting time recorder (Medical Laboratory Inc., Mount Vernon, NY) modified by KEBO-GRAVE (Spånga, Sweden). After addition of 0.1 ml 0.015 M CaCl₂, the clotting time was recorded.

All VIII:C assays were performed against a factor VIII concentrate house standard calibrated against the Second International Standard for Blood Coagulation Factor VIII Human (73/552) obtained from the National Institute for Biological Standards and Control, London, England. Duplicate dilutions at 1/100, 1/200, and 1/400 were tested for each sample and the standard (after predilution to 1 IU/ml) unless otherwise stated.

The test system used for all three assay methods was designed to allow calculations according to the multiple parallel line bioassay principle relating clotting time to log concentration (European Pharmacopoeia, 1971). Calculations of the potencies were performed on a Compucorp 445 Statistician (Computer Design Corporation, Los Angeles, CA), using a special program for factor VIII assays that compensates for any drift of the system by computing a hypothetical standard curve for each sample, depending on its position in the run. All tests were also checked with respect to statistical validity (linearity and parallelism). The coefficient of variation varied between 4% and 8% for the three methods.

Factor VIII Coagulant Antigen

Factor VIII coagulant antigen (VIII:CAg) was determined by the two-site solid-phase immunoradiometric assay described by Holmberg et al. Homologous antibodies were kindly supplied by Prof. I. M. Nilsson, Department for Coagulation Disorders, University Hospital, Malmö, Sweden. Normal human citrate plasma from 20 healthy donors (sex ratio 1:1, A and O blood group ratio 1:1, mean age 35 yr) was used as a standard.

Factor-VIII-Related Antigen

Factor-VIII-related antigen (VIIIR:Ag) was measured with a quantitative electroimmunoassay. Goat antiserum was purchased from Atlantic Antibodies (Scarborough, ME). The VIIIR:Ag assays were standardized against the First British Standard for Blood Coagulation Factor VIII-related Antigen Human for Immunohassay (66/355). Crossed immunoelectrophoresis (XIEP) was performed according to Lamb et al., using 2% agarose (1% Litex type HSB, Denmark, and 1% Bio-Rad low temperature agarose, Richmond, VA) and silver staining, essentially according to Oakley et al.

Heparin

Heparin was analyzed as anti-Xa activity using Coatest/Heparin (KabiVitrum AB, Stockholm, Sweden).

Ionized Calcium

Ionized calcium was measured on the Radiometer Research pH meter PHM64 with a calcium-sensitive electrode (Radiometer F2000) according to the manufacturer’s instructions.

Atomic Absorption Spectrophotometric Analysis

This was performed at Analytica AB (Sollentuna, Sweden) using a Pye Unicam SP1900 atomic absorption spectrophotometer (Cambridge, England). Measurements were made at 422.7 nm, using an air-acetylene flame. Calcium standard solutions were made up in 2% HNO₃. CsCl was added to a final concentration of 0.1% to the standard as well as to the sample dilutions. Since the viscosity of the protein solutions might have an influence on the aspiration of the samples, control measurements were performed after the addition of standard solutions to final concentrations of 0.5 and 1.0 μg/ml. Magnesium, manganese, and zinc were analyzed in similar ways, except for the addition of CaCl₂. Measurements were made at 285.2, 279.5, and 213.9 nm, respectively.

Aluminum, copper, iron, and strontium were determined using a Varian CRA90 carbon rod atomizer and a Pye Unicam SP192 atomic absorption spectrophotometer monitored at 309.3, 248.8, 248.3, and 460.7 nm, respectively. Undiluted samples were tested, and standard additions to 0.02 and 0.04 μg/ml were made for all determinations.

Collection of Blood

Nine pairs of blood were drawn from healthy individuals (8 donors, laboratory staff) into 1 part anticoagulant solution in plastic tubes. Three different types of anticoagulants were used: (A) CPD solution, containing 0.016 M citric acid, 0.089 M trisodium citrate, 0.018 M monosodium phosphate, and 25.5 g/liter dextrose. (B) Heparin 5,000 IU/ml (KabiVitrum AB, Stockholm, Sweden), was diluted in saline to 50 IU/ml. (C) EDTA, 0.05 M, was prepared from Tiritplex III (E. Merck, Darmstadt, Germany) in saline.

Plasma was prepared by centrifugation for 7 min at 7,000 g and used immediately or kept frozen at −70°C before use. The pH of CPD and heparin plasma was 7.6 and 7.7, respectively.
Stability Studies

In the first part of the stability study, plasma samples prepared from blood collected in CPD, EDTA, or heparin solutions as anticoagulants were incubated in plastic tubes at 37°C in a water bath. Samples for VIII:C assays were withdrawn after 0, 2, 4, 6, 24, and 30 hr, treated with Heparsorb, and analyzed immediately by the "Lode one-stage method."

In the second part of the stability study, CPD plasma was incubated at 37°C after addition of heparin and chlorides of divalent metal ions. One part of a solution containing heparin, 50 IU/ml, and metal chloride, 0.06, 0.10, 0.15, or 0.20 M, was added to 9 parts of CPD plasma. Saline or heparin (50 IU/ml) in saline was added in the same proportions to control plasma samples. Incubation and sampling were performed as described above. The experiments with 0.20 M CaCl₂ (and control) were repeated using varying assay techniques as well as after addition of various protease inhibitors (immediately after the centrifugation of the blood).

Separate samples for immunologic characterization were withdrawn at 0 and 24 hr and kept frozen at -70°C until assayed. The level of ionized calcium was measured in CPD, EDTA, and heparin plasma samples and also in samples of CPD plasma after addition of heparin and CaCl₂.

Purification of Factor VIII

Six bottles of Octonativ 500 IU (high purity factor VIII concentrate, KabiVitrum AB, Stockholm, Sweden) were each reconstituted in 5 ml of water. The solution was precipitated by polyethylene glycol (Carbowax 4000, Union Carbide Norden AB, Stockholm, Sweden) at a concentration of 4% (pH 6.8) and kept for 1 hr at room temperature. The supernatant was applied to a 5 × 90 cm column of Sephacryl 30-6B (or Sephacryl S-500) columns (2.6 × 30 cm, flow rate 30 ml/hr). Elution was performed at 37°C in Tris buffer (0.05 M Tris, 0.15 M NaCl, 0.5% HSA, 0.02% azide, pH 7.35) or the same buffer with 2 mM CaCl₂ for heparin plasma. EDTA plasma was incubated for 1 hr at 37°C before chromatography and eluted in Tris buffer with 2 mM EDTA. Three-milliliter fractions were collected and assayed immediately for VIII:C by the Electra method. Samples for determination of VIII:CAg, VIIIR:Ag, HSA, IgG, and heparin were kept frozen at -70°C until examined.

EDTA plasma was also chromatographed after addition of DFP to a final concentration of 2 mM in plasma and 0.1 mM in the elution buffer.

RESULTS

Stability Studies

Three kinds of anticoagulants with different metal-binding abilities were used in the stability studies. The composition of the CPD solution was the same as in standard blood bags, but the ratio anticoagulant:blood was 1:9, resulting in a final concentration of 10.5 mIU citrate in the whole blood. The concentration of ionized calcium in the CPD plasma was 0.07 mM when measured by the calcium-specific electrode. EDTA (final concentration 5 mM) was used as a more potent chelator than citrate, and the level of ionized calcium in the EDTA plasma was below 0.01 mM. When using heparin as anticoagulant, the physiologic level of ionized calcium, approximately 1 mM, was maintained.

A pronounced difference in the stability of VIII:C in plasma at 37°C was observed when these three anticoagulants were used. In EDTA plasma, the VIII:C activity disappeared rapidly, and all activity was lost within 2 hr. The decay of VIII:C in CPD plasma was different. About 50% of the initial VIII:C was inactivated during a first rapid phase. After that period, the inactivation was comparatively slow. The biphasic disappearance curve obtained for CPD plasma is shown in Fig. 1. Transferring the graph onto a log-linear scale revealed a straight line for the first phase, consistent with a first-order reaction. Addition of heparin (final concentration 5 IU/ml) did not have any influence on the decay of VIII:C in CPD plasma.

Since the initial activity of VIII:C in the individual CPD plasma samples varied, a kinetic approach was conceived for the purpose of exploring the question of whether factor VIII was inactivated by enzymes. The graphs obtained by plotting the initial rates of decay (Vo) against the various concentrations of the hypothetical substrate VIII:C [S] or V0 against V0/|S| were not consistent with Michaelis-Menten kinetics.

Gel Filtration Experiments

Blood was collected in warm (37°C) plastic tubes containing CPD, EDTA, or heparin solutions, as described above, and centrifuged immediately at 37°C in a prewarmed rotor at 7,000 g for 7 min. Three-milliliter plasma samples were directly applied to Sepharose CL-6B (or Sephacryl S-500) columns (2.6 × 30 cm, flow rate 30 ml/hr). Elution was performed at 37°C in Tris buffer (0.05 M Tris, 0.15 M NaCl, 0.5% HSA, 0.02% azide, pH 7.35) or the same Tris buffer with 2 mM CaCl₂ for heparin plasma. EDTA plasma was incubated for 1 hr at 37°C before chromatography and eluted in Tris buffer with 2 mM EDTA. Three-milliliter fractions were collected and assayed immediately for VIII:C by the Electra method. Samples for determination of VIII:CAg, VIIIR:Ag, HSA, IgG, and heparin were kept frozen at -70°C until examined.

EDTA plasma was also chromatographed after addition of DFP to a final concentration of 2 mM in plasma and 0.1 mM in the elution buffer.

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VIII:A CALCIUM PROTEIN COMPLEX

Fig. 1. Stability of VIII:C in CPD and heparin plasma at 37°C. VIII:C was determined by the "Lode one-stage assay" and expressed as percent of the initial activity. Each point represents the mean ± SD of at least 6 experiments (3 values at 30 hr). (C) CPD plasma; (O) heparinized CPD plasma; (□) heparin plasma.

Accordingly, the decay of VIII:C could not be identified as an ordinary enzyme-substrate reaction.

When heparin was used as the sole anticoagulant, i.e., at physiologic levels of Ca²⁺, VIII:C was much more stable than in the presence of citrate (Fig. 1). The initial levels of VIII:C in heparin plasma and heparinized CPD plasma were identical (mean 1.2 IU/ml, 10 matched pairs). After 6 hr of incubation, however, the residual activities were 90% and 54%, respectively. This difference, as well as the differences observed after 2 and 4 hr, were statistically significant (p < 0.001 Student's dependent t-test). During further storage, the activity declined in the heparin plasma.

When the influence of Ca²⁺ and the other metal ions was investigated, heparin was added to CPD plasma in order to prevent the coagulation expected to follow the neutralization of citrate by metal ions. As shown in the previous experiments, identical results were obtained for CPD plasma whether heparin was present or not. Recalcification of heparinized CPD plasma prior to incubation resulted in a noticeable stabilization of VIII:C (Fig. 2). When CaCl₂ was added to a final concentration of 20 mM, 95% of the initial activity still remained after 30 hr of incubation. It is noteworthy that the results in Figs. 1 and 2, which represent many sets of experiments and different donors, display a rather small variation that can mainly be attributed to the variation of the assay method.

In order to investigate this Ca²⁺ stabilization further, CPD plasma was stored after the addition of heparin and various amounts of CaCl₂. The concentration of ionized Ca²⁺ in the plasma samples was measured using a calcium-specific electrode. Figure 3 clearly shows that stabilization of VIII:C is achieved when the physiologic level of ionized calcium (about 1 mM) is restored by the recalcification. A similar stability, about 90% residual activity after 6 hr, was observed when blood was collected in heparin alone (Fig. 1).

The influence of other divalent cations on the stability of VIII:C was also investigated. As shown in Table 1, Sr²⁺ prevented activity losses to the same extent as Ca²⁺ when added to a final concentration of 10 mM. Partial stabilization during the first 6 hr was observed for Mg²⁺ and Mn²⁺. Addition of Ba²⁺, Zn²⁺, Fe²⁺, Cu²⁺, Co²⁺, and Ni²⁺ did not protect VIII:C against inactivation.

Sr²⁺, Mg²⁺, and Mn²⁺ were further tested at a final concentration of 20 mM. The residual VIII:C after 24 hr at 37°C was 99%, 51%, and 65%, respectively (one
samples with Heparsorb does not have any influence on an analytical problem, however, which was overcome virtually identical with the results presented in Fig. 2. Furthermore, VIII:C was assessed under the same conditions, but residual VIII:C was followed by other displayed in Fig. 2, were repeated under the same heparin. That VIII:C could be assayed without prior removal of in the presence of such a low level of heparin (1 IU/ml) when the recalcification experiments were performed in our laboratory. Consistent results were also obtained by the use of Heparsorb. According to Hoffman and Meulendijk and Cowan et al., treatment of plasma samples with Heparsorb does not have any influence on VIII:C assays. This was confirmed in a separate study in our laboratory. Consistent results were also obtained when the recalification experiments were performed in the presence of such a low level of heparin (1 IU/ml) that VIII:C could be assayed without prior removal of heparin.

The important question of whether proteases were involved in the inactivation or the stabilization of VIII:C was explored by repeating the experiments shown in Fig. 2 in the presence of various protease inhibitors besides heparin. The inhibitors were added immediately after the centrifugation of the blood. Addition of Trasylol and PMSF (100 KIE/ml and 1 mM in plasma) or DFP (2 mM in plasma) did not change the results at all. S 2581, a very potent inhibitor of thrombin with , was also tested. Although added to such a high concentration (20 μM in plasma) that the clotting times during assaying were prolonged despite high dilutions, the principal results were not affected.

Finally, immunologic characterization of fresh and stored plasma samples were performed. In fresh plasma samples, the same levels of VIII:Ag were found in CPD, heparin, and EDTA plasma, as well as in heparinized CPD plasma with 20 mM added CaCl2. The apparent level of VIII:CaG, however, was about 30% higher in the fresh EDTA plasma than in the other samples. VIII:CaG and VIIIIR:Ag were unaffected by storage at 37°C for 24 hr, except that VIIIIR:Ag in EDTA plasma decreased to about 70% of the initial level. The XIEP patterns for VIIIIR:Ag were identical for the 4 fresh plasma samples, as well as for the samples incubated for 24 hr.

Table 1. Effect of Various Metal Ions on the Stability of VIII:C in Heparinized CPD Plasma

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>No. of Experiments</th>
<th>Percent VIII:C Mean ± SD</th>
<th>Hours of Incubation at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
<td>100 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>4</td>
<td>106 ± 6</td>
<td>6</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>4</td>
<td>108 ± 12</td>
<td>10</td>
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<tr>
<td>Ba²⁺</td>
<td>3</td>
<td>92 ± 8</td>
<td>24</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>3</td>
<td>87 ± 8</td>
<td>0</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>3</td>
<td>84 ± 14</td>
<td>6</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>3</td>
<td>73 ± 8</td>
<td>24</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>3</td>
<td>64 ± 3</td>
<td>24</td>
</tr>
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</table>

VIII:C activity (determined by the "Lode one-stage assay") expressed as percent of the initial activity in heparinized CPD plasma.

Metal Content of Purified Factor VIII Complex

The stability studies clearly demonstrated that inactivation of VIII:C in CPD plasma is related to chelation of Ca²⁺ and possibly some other metal ions. These results suggest that the factor VIII complex is a metallo-protein. In order to identify and quantify any intrinsically bound metal ions, purified factor VIII complex was subjected to metal determination using atomic absorption spectrophotometric analysis.

Before the metal determinations, buffer exchange
was performed in order to remove all unbound or loosely associated metals. The yield of VIII:C in the Sephadex G-25 chromatography step was 80%–90%. The yields of VIII:CAg, VIIIR:Ag, and protein were 90%–100%. The purity of the factor VIII complex (G-25 void volume fractions) was investigated by gradient gel electrophoresis. Figure 4 shows a run on PAA 2/16 gradient gel. Part of the protein appeared at a relative molecular mass of 1–3 × 10^6 daltons, but most of the protein did not penetrate the gel at all. Similar results were obtained on PAA 4/30 gradient gels. Immunologic analysis showed minor contamination by IgM (3%) and fibronectin (1% of the total protein). The content of albumin, Clq, fibrinogen, IgA, and IgG was less than 1% of the total protein. In one experiment, G-25 fractions were collected and assayed individually for VIIIR:Ag and calcium. The elution pattern is shown in Fig. 5. The total recovery was 102% and 89%, respectively. Of the recovered calcium, 58% eluted in the void volume with factor VIII and 42% eluted as non-protein-bound calcium. In following experiments, fractions 2–6 (13–23 ml), containing the factor VIII complex, were pooled and assayed for protein, calcium, and other elements (Table 2). Fractions eluting ahead of the void volume were used as control samples and contained less than 0.05 μg/ml of all assayed elements.

On average, the factor VIII preparations contained 0.18 μg calcium/mg protein or about 1.0 mole calcium/220,000 subunit. The subunit of VIHR was chosen for the calculation of the molar ratio, since VIIIR constitutes the bulk (about 99%) of the factor VIII complex. After incubation of the purified factor VIII complex with EDTA (final concentration 0.01 M) and subsequent buffer exchange on Sephadex G-25, cal-

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**Table 2. Calcium Content of Purified Factor VIII Complex**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein (mg/ml)</th>
<th>Ca (μg/ml)</th>
<th>Ca (Mole/220,000 Daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>0.8</td>
<td>0.15</td>
<td>1.1</td>
</tr>
<tr>
<td>No. 2</td>
<td>1.1</td>
<td>0.14</td>
<td>0.7</td>
</tr>
<tr>
<td>No. 3</td>
<td>0.9</td>
<td>0.20</td>
<td>1.2</td>
</tr>
<tr>
<td>Mean</td>
<td>0.9</td>
<td>0.16</td>
<td>1.0</td>
</tr>
<tr>
<td>EDTA-inactivated preparation</td>
<td>0.8</td>
<td>&lt;0.05</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Control (buffer fractions from each experiment)</td>
<td>—</td>
<td>&lt;0.05</td>
<td>—</td>
</tr>
</tbody>
</table>

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Fig. 4. Polyacrylamide gel electrophoresis on PAA 2/16 gradient gel in Tris-borate buffer, pH 8.4, for 18 hr at 125 V. Samples: (A) human serum, (B) human serum diluted 1:5, (C) factor VIII preparation (20 μg), (D) high molecular weight calibration proteins. (1) α2-macroglobulin, mol wt 820,000. (2) Thyroglobulin, mol wt 669,000. (3) Ferritin, mol wt 440,000. (4) Catalase, mol wt 232,000.

Fig. 5. Gel filtration of purified factor VIII complex on Sephadex G-25 Superfine in ultrapure Tris-acetate buffer. Ten milliliters of purified factor VIII complex containing about 3,000 U VIIIR:Ag was applied.
cium could not be detected in the protein fractions and all the coagulant activity was lost.

No significant levels of the other analyzed metals were found in factor VIII. Manganese, strontium, and zinc were below 0.02, and aluminium, magnesium, copper, and iron were below 0.05 mole/subunit.

Gel Filtration Experiments

The metal analysis furnished proof that calcium is closely associated with factor VIII. To support the hypothesis that native factor VIII circulates in blood as a calcium-linked protein complex, gel filtration experiments were performed on fresh, warm (37°C) plasma samples containing various anticoagulants. The collection, centrifugation, and gel filtration steps were all carried out at 37°C. Representative elution profiles obtained for heparin, CPD, and EDTA plasma by gel filtration on Sepharose CL-6B are shown in Fig. 6. All fractions were assayed for VIII:C, VIII:CAg, and VIIIR:Ag. The factor-VIII-related activities eluted together when heparin or CPD plasma was chromatographed. The main part eluted in the void volume fractions, and the last trailing part eluted ahead of the main protein peak. Only trace amounts of VIII:C and VIII:CAg could be detected in fractions eluting after IgG.

The yields of VIII:C, VIII:CAg, and VIIIR:Ag (in the fractions eluting between 50 and 100 ml) were 78%, 85%, and 68% for heparin plasma and 57%, 57%, and 67% for CPD plasma. The same type of chromatography pattern was observed when the gel filtration was performed on Sephacryl S-500 columns. With Sephacryl, however, the recovery of VIII:C and VIII:CAg from CPD plasma was very poor (10%–40%), indicating adsorption to the gel matrix. In 9 separate chromatography experiments for each of heparin and CPD plasma, the factor-VIII-related activities consistently eluted together, displaying elution profiles almost identical with those shown in Fig. 6.

When CPD plasma was stored for 22 hr at room temperature, 44% of the VIII:C activity was lost. When this plasma sample was chromatographed, the

![Fig. 6. Gel filtration on Sepharose CL-6B at 37°C of plasma prepared in various anticoagulants. The final concentration in whole blood of heparin, citrate, and EDTA was 5 IU/ml, 10.5 mM, and 5 mM, respectively. Heparin and CPD plasma samples were applied to the column immediately after centrifugation. Heparin plasma was chromatographed in elution buffer containing 2 mM CaCl₂. The EDTA plasma was incubated for 1 hr at 37°C before chromatography, and then eluted in buffer containing 2 mM EDTA. Fractions containing EDTA were not assayed for VIII:C. The bars denote the elution volumes for IgG (G) and albumin (A). (Δ−−−Δ) VIII:C, (Δ−−−Δ) VIII:CAg, (Φ−−−Φ) VIIIR:Ag, (−−−−−−) A₂₈₀.](image-url)
remaining VIII:C eluted together with VIII:CAg and VIIIR:Ag in the void volume. About 15% of recovered VIII:CAg eluted later at a position between IgG and albumin.

Gel filtration of EDTA plasma in the presence of 2 mM EDTA resulted in a complete dissociation of VIII:CAg and VIIIR:Ag (Fig. 6). Similar results were obtained in the presence of EDTA plus DFP. VIIIR:Ag eluted in the void volume and 89% was recovered. VIII:CAg eluted along with albumin, but the recovery in these fractions was only 31%. After Sephacryl S-500 chromatography, hardly any VIII:CAg was detected in the EDTA plasma fractions, but about 50% could be recovered from the column by elution with 1 M NaCl. These results indicate that separated VIII:CAg easily adsors to the gel matrix. The apparent elution volume for the recovered VIII:CAg could not be used to determine the molecular size of dissociated VIII:CAg, since this material might also have been delayed by the adsorption effects.

**DISCUSSION**

Recent reviews of factor VIII illustrate the enormous amount of biochemical and clinical data, often contradictory, that await clarification. The elusive nature of this molecular complex is partly ascribed to the pronounced lability of the coagulant part. In agreement with previous reports, we have found that VIII:C in citrated plasma deteriorates during storage in a biphasic manner. The fact that addition of heparin and various protease inhibitors does not protect VIII:C against this inactivation at all, as well as the information gained by the kinetic analysis, indicate that factor VIII is not inactivated by a proteolytic mechanism but rather by the chelation of essential metal ions.

The biphasic decay of VIII:C in citrated plasma is intricate and may indicate consecutive decay reactions. The chelation of Ca$^{2+}$ appears to induce transformation of the native VIII:C into a fairly stable intermediate form with reduced ability to express coagulant activity. However, the possibility that the coagulant entity is heterogeneous with respect to Ca$^{2+}$ binding and stability, cannot be entirely ruled out. The biphasic decay might reflect parallel inactivation of two molecular forms. Theoretical disappearance curves were constructed for these two models and compared with the observed data. However, both of the simulated graphs coincided with those obtained experimentally. Thus, the two hypotheses could not be rejected, neither could any one be selected as superior by this kinetic approach.

Collection of blood in heparin instead of chelating anticoagulants or neutralization of citrate by the addition of Ca$^{2+}$ to heparinized CPD-plasma rendered VIII:C noticeably stable. At physiologic levels of ionized calcium, VIII:C was almost completely stable during incubation of plasma for 6 hr at 37°C. Prolonged stabilization was achieved at higher concentrations of ionized calcium. These results confirm the findings of Rock et al., who showed that VIII:C was more stable in heparinized plasma than in citrated plasma. Stibbe et al. observed that the rate of decay was slowed down when Ca$^{2+}$ was added to resin plasma, but they did not restore physiologic Ca$^{2+}$ levels. For that reason, they never achieved a complete stabilization.

Several metal ions may protect VIII:C against in vitro loss of activity. In this study, Ca$^{2+}$, Sr$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$ prevented the inactivation to a varying degree. The same general conclusion was drawn by Weiss, who studied the influence of various divalent cations on the stability of factor VIII by adding EDTA-metal complexes to resin or citrate plasma.

Manganese has been reported to stabilize and even restore coagulant activity from inactivated factor VIII. However, this enhancing effect of manganese may have been an artifact. Early experiments in our laboratory showed that Mn$^{2+}$ itself shortened the clotting times when VIII:C was assessed by the ordinary assay methods. Using the modified assay as described in this report, manganese only partially protected VIII:C against inactivation.

The possibility that the high residual level of VIII:C after incubation in the presence of Ca$^{2+}$ is due to activation is very unlikely since addition of protease inhibitors did not have any influence on the stabilization achieved by the recalcification.

Although this investigation clearly demonstrates that chelating agents inactivate VIII:C, and furthermore, that Ca$^{2+}$ and some other metal ions may protect VIII:C against this inactivation, stability studies cannot furnish proof of any intrinsic metal ion being essential to the preservation of structure and function. The metal analysis, however, revealed that highly purified factor VIII complex contains calcium at a ratio of about 1.0 mole/220,000 daltons. The preparations of factor VIII used for the metal determinations were extensively purified in the presence of citrate. Thus, the estimated content of calcium reflects only the high affinity Ca$^{2+}$ binding properties of factor VIII. Since citrate partially inactivates the coagulant activity in plasma, total preservation of the structural integrity requires further accessible Ca$^{2+}$. The intrinsic Ca$^{2+}$ found in the purified factor VIII is evidently bound tightly enough to withstand chelation by citrate but may be readily removed upon exposure to EDTA,
with concomitant loss of the coagulant activity. Factor VIII has not been proved previously to be a Ca$^{2+}$- dependent metallo-protein complex. However, factor V, which in many respects is similar to factor VIII, has been shown to contain 1 mole Ca$^{2+}$/mole factor V (bovine), with a molecular mass of 330,000 daltons.

The gel filtration experiments furnished further support to the concept that native factor VIII is a molecular complex that is comprised of separate entities that are linked together by Ca$^{2+}$. In fresh heparin plasma and CPD plasma, all the factor-VIII-related activities eluted together as large protein complexes, whereas VIII:CaG and VIIIIR:Ag were completely dissociated in EDTA plasma. These data are in good agreement with the results reported for citrated and heparinized plasma by Bolhuis et al. The presence of high and low molecular forms in heparinized plasma, as found by Rock et al., could not be demonstrated.

In conclusion, the present investigation provides evidence that human factor VIII circulates as a Ca$^{2+}$-linked protein complex under physiologic conditions. In the presence of citrate, the coagulant activity is partially lost, but the factor VIII complex is not dissociated. When purified from citrated plasma, the protein complex still retains intrinsic Ca$^{2+}$ and is able to express about 30% coagulant activity, as reflected by the VIII:C/VIIIIR:Ag ratio. Upon exposure to EDTA, however, Ca$^{2+}$ is removed, the complex is decomposed, and all coagulant activity is lost. Consequently, it is reasonable to assume that there are different binding sites for Ca$^{2+}$ in the factor VIII complex. The molar stoichiometry of the intrinsic Ca$^{2+}$, estimated at about 1.0 mole/mole subunit of VIIIIR, is merely a mathematical conception. The current data do not provide any information about the location of the Ca$^{2+}$-binding sites. Probably most of the Ca$^{2+}$ originates from the carrier protein (VIIIIR), since this part of the protein complex constitutes about 99% of the total protein mass. Neither the size nor the distribution of the large VIIIIR multimers are affected by the presence or absence of ionized calcium. The Ca$^{2+}$-binding sites might, however, be of importance for the expression of von Willebrand activity, i.e., interactions with subendothelium and platelets. Studies by Sakariassen et al. demonstrated that various steps of platelet interaction with subendothelium require Ca$^{2+}$ and factor VIII.

A most important function of the intrinsic Ca$^{2+}$ can be inferred from the current study, that is, as a stabilizer of the quaternary structure of the factor VIII complex. The conclusion that the coagulant part is linked by Ca$^{2+}$ bridges to the carrier protein is in good agreement with the general idea that the factor-VIII-related components interact through noncovalent bonds. It also complies with the proposed role of VIIIIR as a stabilizer of VIIIIR:C. Furthermore, it is likely that the coagulant moiety in itself is dependent on Ca$^{2+}$ to maintain its molecular structure and coagulant function. Recent reports indicate the presence of subunits in purified VIIIIR:C. Fass et al. suggested that the three chains (166,000, 130,000, and 76,000 daltons) seen in SDS-PAGE analysis of porcine VIIIIR:C might be associated by Ca$^{2+}$ bridges. Finally, Ca$^{2+}$ might be essential to the regulatory function exerted by VIIIIR:C in the factor X activation step during coagulation.

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Human factor VIII: a calcium-linked protein complex

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