Studies on human antihemophilic factor. Evidence for a covalently linked subunit structure.

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Research Article

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Studies on Human Antihemophilic Factor

EVIDENCE FOR A COVALENTLY LINKED SUBUNIT STRUCTURE

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ABSTRACT When purified antihemophilic factor (Factor VIII) was rechromatographed on 4% agarose in 0.15 M NaCl or 1.0 M NaCl, a single protein peak, containing both procoagulant activity and von Willebrand factor activity, as defined by ristocetin-induced platelet aggregation, was eluted in the void volume. Purified Factor VIII immediately lost about 30% of its procoagulant activity when dissolved in 0.25 M $CaCl_2$, and when rechromatographed on 4% agarose in 0.25 M $CaCl_2$, the protein peak and von Willebrand factor activity remained coincident in the void volume; however, most of the remaining procoagulant activity was eluted after the void volume. The elution position of Factor VIII procoagulant activity from 4% agarose in 0.25 M $CaCl_2$, and hence its apparent molecular weight, varied with the protein concentration applied to the column; at low protein concentrations it was eluted close to the inner volume. Yet on Sephadex G-200 in 0.25 M $CaCl_2$, the protein and procoagulant activity were eluted together in the void volume. These observations suggested that the Factor VIII procoagulant activity was not eluting according to size or shape, but was adsorbing to some extent to the agarose. Isolated activity peak material from the 0.25 M $CaCl_2$ columns contained protein and had a typical ultraviolet spectrum. Even at high concentrations, the protein contained no thrombin, Factors IX, X, or Xa activity, or detectable phospholipid. In addition to Factor VIII procoagulant activity, which could be inactivated by a human antibody to Factor VIII, the activity peak protein also contained von Willebrand factor activity. Like native Factor VIII and the void volume protein, the activity peak contained protein that did not enter a sodium dodecyl sulfate 5% polyacrylamide gel in the absence of reducing reagent. After reduction of disulfide bonds, several subunits ranging from 195,000 to 30,000 daltons were observed. These results indicate that the protein in the shifted Factor VIII procoagulant activity peak is large and that its anomalous elution pattern from 4% agarose in 0.25 M $CaCl_2$ results from interaction with the agarose. The Factor VIII-like properties of the activity peak protein and its electrophoretic pattern on sodium dodecyl sulfate gels suggest that it is a species of Factor VIII modified by proteolytic cleavage. These results allow an interpretation that is different from the recently proposed "carrier protein-small active subunit" hypotheses for the structure-function relationships of the Factor VIII molecule.

INTRODUCTION

Human antihemophilic factor (Factor VIII) eluted near the void volume when plasma or Factor VIII concentrates were chromatographed on 2–4% agarose in dilute, neutral buffers (1–7). The $s_{25,w}$ value of 16.3 for Factor VIII in the potent dispersing reagent guanidine hydrochloride appeared to rule out a noncovalently linked subunit structure (4). Recent studies of purified Factor VIII showed that it is a glycoprotein with a molecular weight of about one million (6) and that it is composed of disulfide-bound subunits which contain carbohydrate (5–8). Sodium dodecyl sulfate gel electrophoresis, sedimentation equilibrium ultracentrifugation, and isoelectric focusing experiments performed in our laboratory (7) indicated that these covalently linked subunits are in fact identical with respect to size and electrical charge. In the absence of reducing agents such as β-mercaptoethanol, none of the above studies reported any evidence for small dissociable subunits, even in the presence of dispersing reagents such as high concentrations of urea,

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guanidine hydrochloride, or sodium dodecyl sulfate. Furthermore, in most of the above investigations, some idea of the purity of the Factor VIII preparation under study was provided.

Despite these biochemical studies of highly purified preparations of Factor VIII, debate about the molecular structure of Factor VIII continues. Several investigators (9–16) have recently reported that Factor VIII activity in plasma or plasma concentrates can be dissociated by chromatography in certain high ionic strength buffers, particularly 0.25 M $CaCl_2$, to yield a high molecular weight "carrier protein" and a low molecular weight "active subunit." The carrier protein eluted in the void volume while the small active subunit containing the procoagulant (PC)[1] activity eluted later. The observation that both purified Factor VIII and the void volume protein from the salt dissociation studies corrected the platelet defect in von Willebrand's disease led to the suggestion that the carrier protein contained the von Willebrand factor (vWF) activity (13–20). These studies have been hampered by an absence of criteria of purity and the lack of biochemical characterizations of the protein(s) associated with the PC or vWF activities.

Using electrophoretically and immunologically homogeneous Factor VIII, we have studied its chromatographic behavior in 0.15 M NaCl, 1.0 M NaCl, and 0.25 M $CaCl_2$. Evidence is presented which is not consistent with the "carrier protein-active subunit" hypothesis for Factor VIII structure. Our results, instead, support a covalently linked subunit structure for Factor VIII and suggest that the molecule has both PC and vWF activities.

METHODS

Reagents. Reagent grade chemicals were used without further purification. Except for dissociation studies, 0.1 M ε-aminocaproic acid and 0.02% sodium azide were used routinely in all buffers. All solutions were buffered to pH 7.35 with 0.05 M Tris hydrochloride except in specifically noted instances.

Protein concentrations. These were estimated by the absorbance at 280 nm with a scattering correction based on the absorbance at 320 nm; concentrations determined by this method were expressed in absorbance units. For comparison, the method of Lowry et al. (21) was also used.

Factor VIII assay. As previously described in detail (7), Factor VIII PC activity was measured by the kaolin-activated partial thromboplastin time, using hemophilic substrate plasma with < 1% Factor VIII activity. The presence of high concentrations of NaCl or $CaCl_2$ in samples to be tested for Factor VIII activity necessitated some special procedures. To reduce the salt concentration to the optimal range, one part of each sample from the 1.0 M NaCl column was diluted with five parts 0.05 M Tris hydrochloride containing no sodium chloride. Subsequent dilutions were performed with 0.15 M NaCl–0.05 M Tris. The Factor VIII activity in units per milliliter was computed from a reference curve obtained by assaying serial dilutions of a National Institutes of Health (NIH) standard reference plasma (0.76 U of Factor VIII PC activity per ml). To analyze Factor VIII samples in 0.25 M $CaCl_2$, one must both dilute the sample and change the order of reagent addition. The assay was performed as follows: one part sample was diluted with five parts 0.15 M NaCl–0.05 M Tris (if serial dilutions were desired the sample was diluted further with 0.15 M NaCl-0.05 M Tris-0.043 M $CaCl_2$) and warmed to 37°C for 90 s; 0.1 ml of this solution, which now contained 0.043 M calcium chloride, was then added to a prewarmed (for 90 s) mixture of 0.1 ml 0.15 M NaCl-0.05 M Tris, 0.1 ml Thrombofax (Ortho Diagnostic Inc., Raritan, N. J.) and 0.1 ml of kaolin-activated hemophilic substrate, and the time required to form a visible clot was recorded. This end point was generally less distinct than the end point with the standard order of additions. The Factor VIII activity in units per milliliter was computed from a reference curve obtained by diluting and assaying the NIH reference plasma (0.76 U/ml) in 0.15 M NaCl-0.05 M Tris-0.04 M $CaCl_2$ by the same method as outlined above. The clotting times obtained for the reference plasma dilutions were considerably longer and the slope of the reference curve (log percent dilution versus clotting time) was less than when the assays were performed in the usual order.

Factor IX, X, Xa assays. Factor IX assays were performed by a one-stage partial thromboplastin time using kaolin-activated Factor IX-deficient substrate plasma (22). Factor X assays were done by a standard method (23), using Factor X-deficient plasma. The Factor Xa assay was performed in the same way as the Factor X assay, except that cephalin alone was used instead of Russell viper venom (Burroughs Wellcome & Co., Tuckahoe, N. Y.) and cephalin. In instances where the solution to be assayed contained 0.25 M $CaCl_2$, the sample was diluted and the assay modified as described above for Factor VIII in the presence of 0.25 M $CaCl_2$.

Thrombin assay. Human fibrinogen (Kabi, Stockholm) was made to 5 mg/ml in 0.15 M NaCl-0.05 M Tris; and dilutions of purified human thrombin (supplied by Dr. D. L. Aronson) were made in the same buffer. After warming both solutions to 37°C, clotting times were measured by adding 0.05 ml of each thrombin dilution to 0.2 ml of the fibrinogen solution and observing for gel formation. A standard curve from which the thrombin content of test samples could be estimated was constructed with final thrombin concentrations of 40, 20, 10, 5, 2.5, and 1.25 NIH U/ml.

Inorganic phosphate determinations. These were performed by the ascorbic acid-molybdate method (24, 25). A standard curve was prepared from known concentrations of K_2HPO_4. To avoid contamination by phosphate, all glassware used for these experiments was new or was thoroughly washed with concentrated nitric acid.

Purification of normal Factor VIII. The starting material in these experiments was either glycine-precipitated concentrates (method IV, Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) or the intermediate-purity material of Newman et al. (26) as supplied by the American National Red Cross. The high purity Factor VIII of Newman et al. (26) was prepared by their method with the following modifications: the glycine-precipitated Factor VIII was introduced into the purification scheme at the 0.02 M Tris-0.2 M ε-aminocaproic acid extraction step; the

[1] *Abbreviations used in this paper:* CTA, Committee on Thrombolytic Agents; NIH, National Institutes of Health; PC, procoagulant; SDS, sodium dodecyl sulfate; vWF, von Willebrand factor.

intermediate purity material was not subjected to adsorption by aluminum hydroxide gel. A bentonite adsorption step was used to decrease fibrinogen content before precipitation with polyethylene glycol. This adsorption was performed by adding bentonite (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 10 mg/ml to the intermediate-purity Factor VIII solution. This mixture was then stirred slowly for 10 min at room temperature and centrifuged in polycarbonate bottles at 6,000 *g* for 10 min at 20°C. The remaining precipitation steps described by Newman et al. (26) were then carried out on the supernatant fluid. The final precipitate was dissolved in 0.15 M NaCl-0.05 M Tris-0.1 M ε-aminocaproic acid and 0.02% azide to a final protein concentration of 10–20 mg/ml. In some instances the starting material was high-purity Factor VIII concentrate prepared by the American National Red Cross (generously supplied by Dr. M. Wickerhauser and Dr. Y. L. Hao). Before use, this Factor VIII concentrate was reconstituted with 0.2 M ε-aminocaproic acid to give a protein concentration of about 20 mg/ml.

High-purity Factor VIII concentrate was then further purified by gel filtration in 0.15 M NaCl-0.05 M Tris buffer at room temperature on 4% agarose (Biogel A15m, Bio-Rad Laboratories, Richmond, Calif.) in a siliconized glass column (4 × 38 cm). The sample was applied directly to the top of the agarose and washed into the column with 3 vol of buffer. The volume of high-purity Factor VIII applied to the column was always about 2–3% of the column volume. The flow rate averaged 20 ml/h and 7-ml fractions were collected in plastic tubes. The fractions eluting in the void volume were pooled and assayed for protein and Factor VIII activity. Factor VIII was precipitated by cooling the solution in an ice bath and adding freshly prepared, ice-cold 40% polyethylene glycol (Carbowax 4000, Union Carbide Corp., South Charleston, W. Va.) in 0.15 M NaCl-0.05 M Tris to give a 12% final polyethylene glycol concentration. The solution was stirred for 15 min and then left overnight in an ice bath. The Factor VIII was isolated by centrifugation at 4°C and 10,000 *g* and resuspended in 0.15 M NaCl-0.05 M Tris to a final protein concentration of at least 3 mg/ml. This material, termed "ultra-high purity Factor VIII," was homogeneous by gel electrophoresis as well as by immunodiffusion and immunoelectrophoresis against unadsorbed rabbit antiserum. The Factor VIII samples were stored at −20°C as 0.05- to 1.0-ml aliquots until needed. Yield data and degree of purification at each step have been published previously from this laboratory (7).

Electrophoresis. Sample preparation and polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was carried out essentially as described earlier (7). Gels were stained for protein or carbohydrate, destained, and stored as previously described (7).

Chromatography studies. Ultra-high purity Factor VIII was rechromatographed on 4% agarose in the following buffers: 0.15 M NaCl-0.05 M Tris, 1.0 M NaCl-0.05 M Tris, or 0.25 M $CaCl_2$-0.01 M NaCl-0.05 M Tris, each of which was pH 7.35. Columns were calibrated with Blue Dextran (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and pumped negatively with a peristaltic pump to maintain constant flow. Plastic columns of different dimensions (0.9 × 11, 0.9 × 26, 0.9 × 55, and 2.5 × 98 cm) were used for these experiments; in each instance the sample volume was 2–3% of the column volume, and the fractions were approximately the same volume as the applied sample. All of these columns gave satisfactory resolution of the protein peak from the Factor VIII procoagulant activity peak when 0.25 M $CaCl_2$ was used in the eluting buffer. In addition, Factor VIII was chromatographed on Sephadex G-100 and Sephadex G-200 (Pharmacia Fine Chemicals, Inc.) in 0.25 M $CaCl_2$ on 0.9 × 12-cm and 0.9 × 26-cm plastic columns. The chromatography experiments were performed as follows: Samples of electrophoretically pure Factor VIII in 0.15 M NaCl-0.05 M Tris were thawed at 37°C, diluted to the desired absorbance at 280 nm with the same buffer, and the salt concentration adjusted with solid NaCl or $CaCl_2$, depending on the chromatographic conditions. Aliquots of each sample were saved for assays; the remainder was applied to the top of the column bed. The column eluent was monitored for protein, Factor VIII activity and von Willebrand activity as described elsewhere in this section. Fractions beneath the peaks of interest were pooled for concentration; each pool was dialyzed once against 2,000 times its volume of distilled water at 4°C for 18–24 h. The samples were then quick-frozen, lyophilized to dryness, and stored at −20°C for future analysis by SDS-gel electrophoresis.

Antibody experiments. 10 parts of plasma from a hemophilic patient with a high titer of an acquired Factor VIII inhibitor were adsorbed with one part aluminum hydroxide gel, stirred for 5 min at room temperature, and centrifuged at 6,000 *g*. Samples of purified Factor VIII or various column fractions were then incubated at 37°C in a ratio of 1 part adsorbed plasma: 10 parts Factor VIII sample for time periods specified in the Results section. The Factor VIII activity of the incubation mixtures was then compared to that of control incubation mixtures which contained 1 part 0.15 M NaCl-0.05 M Tris buffer: 10 parts Factor VIII sample.

Plasmin-digested Factor VIII. A terminal plasmin digest of purified Factor VIII (27) was prepared by incubating 1 ml of Factor VIII (5 absorbance U at 280 nm) with 0.5 CTA (Committee on Thrombolytic Agents) U of purified (28) human plasminogen (22 CTA U/mg) and 5 Ploug U of urokinase at 37°C for 24 h. The reaction was monitored by Factor VIII activity assays and by SDS-gel analysis of the proteolytically degraded and β-mercaptoethanol-reduced Factor VIII.

Platelet studies. Siliconized glassware or plasticware was used throughout these studies. Venous blood was collected by a two-syringe technique and anticoagulated with 1 part 3.2% sodium citrate: 9 parts blood. Platelet-rich plasma was prepared by centrifuging the anticoagulated blood for 10 min at 300 *g* and 25°C. A platelet pellet was prepared by recentrifuging the platelet-rich plasma for 10 min at 10,000 *g* and 25°C. The platelet pellet was then washed three times with a 4-ml vol of 0.15 M NaCl-0.05 M Tris-0.02 M EDTA and resuspended in 0.15 M NaCl-0.05 M Tris. The platelet count was adjusted to ~50,000/mm^3 with the same buffer. Platelet aggregation studies were performed using a Chrono-Log aggregometer (Chrono-Log Corp., Broomall, Pa.) attached to a Heath Servo-Recorder (Heath Co., Benton Harbor, Mich.). Ristocetin (1,040 *Bacillus subtilus* bactericidal U/mg, kindly supplied by Dr. Grant H. Barlow, Abbott Laboratories, Chicago, Ill.) was dissolved in 0.15 M NaCl-0.05 M Tris to a final concentration of 15 mg/ml. Before each aggregation assay, the recorder was adjusted to 90% transmittance with buffer and to 10% transmittance with the platelet suspension. To 0.4 ml of platelet suspension, 0.05 ml of Factor VIII test sample was added; after 1 min had elapsed for base-line stabilization, 0.05 ml of ristocetin solution was added and the transmittance was recorded until it became constant.

To compute the vWF activity, the maximum slope of percent transmittance versus time was determined and taken

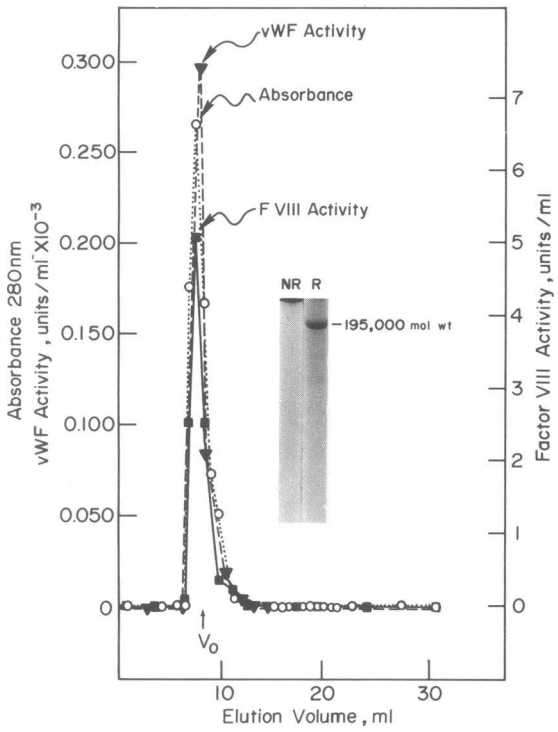


FIGURE 1 4% agarose gel filtration of highly purified Factor VIII in 0.15 M NaCl (column size: 0.9 × 26 cm). The protein, PC activity and vWF activity eluted in the void volume. The protein in the peak was concentrated and analyzed nonreduced (NR) and reduced (R) by SDS-5% gel electrophoresis. No protein entered the nonreduced gel; after reduction a single band of 195,000 daltons was observed.

as the initial velocity (v) of platelet aggregation. By using serial dilutions of pure native Factor VIII in 0.15 M NaCl-0.05 M Tris, a standard curve was constructed for $1/v$ versus 1/[Factor VIII] (as determined from the absorbance at 280 nm). Up to final Factor VIII concentrations of 0.01 mg/ml, the double reciprocal plot was linear. From purifications of Factor VIII from whole plasma in this laboratory, it was estimated that the Factor VIII protein contributes about 0.001 absorbance U to the absorbance at 280 nm of whole plasma. Therefore, 1 U of vWF activity, which has been defined as that contained in 1 ml of fresh, normal plasma (20), is also equivalent to the activity provided by 1 ml of purified native Factor VIII protein with an absorbance of 0.001. Obviously this estimation is fraught with potential error, but it provides a rough guideline. If the Factor VIII protein is inactive or inactivated, it still retains virtually full vWF activity, whereas it has little or no PC activity (27). This fact alone could account for the discrepancy between the units of vWF activity and the units of PC activity in 0.001 absorbance U of purified Factor VIII as arbitrarily defined by us. In addition, the difficulties in absolutely quantitating PC activity or vWF activity also probably contribute to the difference between the two activities per mass of purified protein. To compute vWF activity in units per milliliter, $1/v$ was determined for 0.05 ml of the test sample. The reciprocal of the concentration of purified native Factor VIII required to produce the $1/v$ value for the test sample was then read from a standard curve and converted to vWF units per milliliter, by using the above relationship that 0.001 absorbance U of Factor VIII protein is responsible for the 1 U of vWF activity in 1 ml of fresh, normal plasma. If necessary, the test sample was diluted to obtain $1/v$ values on the linear portion of the double reciprocal plot for the Factor VIII standard. Specific vWF activity was defined as the vWF activity of a sample divided by its absorbance at 280 nm.

RESULTS

The Factor VIII used in the studies reported here had a specific activity of about 25 U/absorbance U at 280 nm and a specific vWF activity of about 1,000 U/absorbance U of purified Factor VIII protein. The ultra-high purity Factor VIII gave a single immunoprecipitin line when tested against unadsorbed rabbit antiserum by double diffusion or immunoelectrophoresis. In addition, when purified nonreduced Factor VIII was analyzed by SDS-gel electrophoresis, only the top of the gel stained for protein and no other bands were seen; after reduction by β-mercaptoethanol, a major protein band of 195,000 daltons and two or three very minor bands ranging from 180,000–160,000 daltons were observed. These latter bands, which were frequently observed when purified Factor VIII preparations were reduced and analyzed on overloaded SDS gels, have been identified (27) as early degradation intermediates of the 195,000-dalton subunit when Factor VIII was digested by plasmin. It is very difficult to isolate native Factor VIII without minor amounts of plasmin-degraded Factor VIII since both the native and minimally degraded forms of Factor VIII are similar in size and elute in the void volume from 4% agarose.

Chromatography of Factor VIII in 0.15 M NaCl. Fig. 1 shows the pattern observed when ∽0.4 absorbance U of purified Factor VIII was rechromatographed on 4% agarose in 0.15 M NaCl. A single sharp peak of protein with both PC and vWF activities was eluted in the void volume. Both the PC and vWF activities were proportional to the protein concentration of this peak. To the right of the chromatogram are SDS-5% polyacrylamide gel patterns of the nonreduced and reduced void volume protein. In the absence of reducing reagent, only the very top of the gel stained for protein and no bands entered the SDS 5% gel, which excludes proteins with molecular weights greater than about 400,000 daltons. After reduction by β-mercaptoethanol, a major band corresponding to a molecular weight of 195,000 daltons was always observed; occasionally two minor bands of ∼ 160,000–130,000 daltons were observed and when present, these constituted less than 5% of the total protein applied.

Chromatography of Factor VIII in 1 M NaCl. Since PC activity has been reported to dissociate from the void volume protein when partially purified Factor VIII is chromatographed on 4% agarose in 0.8–1 M NaCl (11, 14), the effect of 1 M NaCl on purified Factor VIII was examined. Fig. 2 shows that the elution profile was the

same as that observed in 0.15 M NaCl when the same amount of protein was applied. The protein as well as the PC and vWF activities eluted as a single sharp peak in the void volume. Before reduction, the protein in the void volume fractions did not enter a SDS 5% gel; after reduction, however, a single band corresponding to the 195,000-dalton subunit of the native molecule was observed. No lower molecular weight bands were observed on the SDS gels before or after reduction.

Chromatography of Factor VIII in 0.25 M $CaCl_2$. When approximately 2.5 absorbance U of purified Factor VIII was rechromatographed on 4% agarose in 0.25 M $CaCl_2$, the results were very different from those obtained in 0.15 or 1.0 M NaCl. As shown in Fig. 3, a protein peak similar to that observed at either concentration of NaCl eluted in the void volume while most of the PC activity eluted in later fractions. The recoveries of protein and PC activity from the $CaCl_2$ columns were about 80% and 70% with respect to the applied sample made to 0.25 M in $CaCl_2$. There was no loss of procoagulant activity with time in the aliquot of the applied sample assayed at the beginning and end of each chromatography experiment. In general, the bulk of the PC activity eluted in a region of the chromatogram where no protein peak could be detected by absorbance at 280 or 210 nm, by

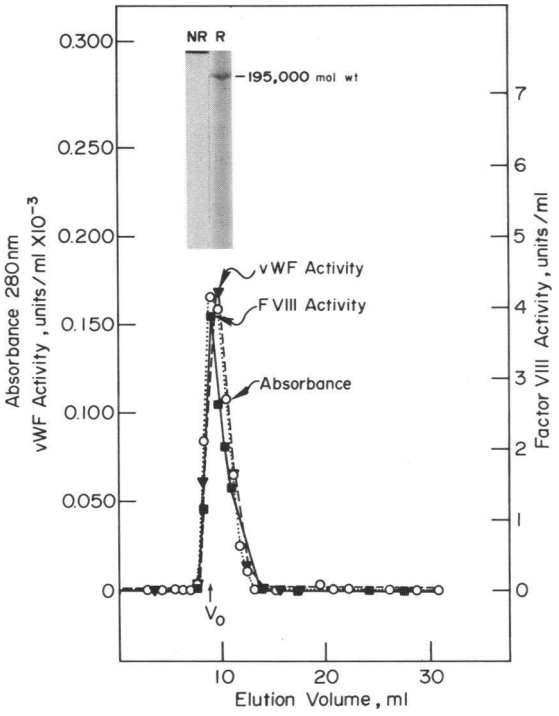


FIGURE 2 4% agarose gel filtration of highly purified Factor VIII in 1.0 M NaCl (column size: 0.9 × 26 cm). The protein, PC activity and vWF activity eluted in the void volume. The SDS-5% gel electrophoretic patterns of the nonreduced (NR) and reduced (R) protein are identical to those in Fig. 1.

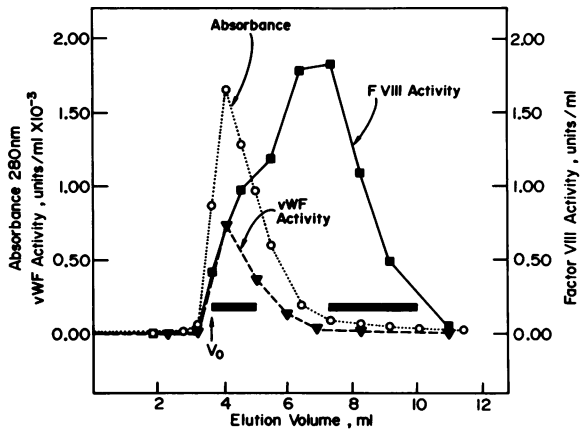


FIGURE 3 4% agarose chromatogram of highly purified Factor VIII in 0.25 M $CaCl_2$ (column size: 0.9 × 11 cm). The protein peak and the vWF activity eluted in the void volume, but the PC activity eluted later. The horizontal bars indicate fractions which were pooled for subsequent study.

the Lowry method or by fluorescence. Most of the vWF activity eluted with the void volume protein peak, although vWF activity could also be detected beneath the PC activity peak. For example, in the chromatogram in Fig. 3, vWF activity is expressed on a scale 10^3 times smaller than protein absorbance. Without concentration, the vWF activity of the fraction eluting at 8 ml is about 17 U/ml, or 17 times that contained in 1 ml of normal plasma. Throughout the elution profile, vWF activity was proportional to the absorbance at 280 nm. Thus, contrary to the reports of others (13, 14), vWF activity could also be easily demonstrated in the PC activity peak, particularly after fractions beneath the PC activity peak had been pooled as indicated by the horizontal bar in Fig. 3, dialyzed against water, lyophilized to dryness, and reconstituted in 0.15 M NaCl-0.05 M Tris buffer to about one-tenth the original volume of the pool. Finally, it should be mentioned that chromatography in the presence of 10% α-methyl-D-glucopyranoside was tried in case the PC activity peak contained a glycoprotein with carbohydrate side chains that interacted with the agarose; however, its use did not alter the elution profile of Factor VIII in either $CaCl_2$ or NaCl.

Effect of protein concentration on the chromatography of Factor VIII in 0.25 M $CaCl_2$. To determine whether the shifted PC activity peak eluted at a constant position, and therefore, as a function of its size and shape, the effect of the protein concentration of the applied sample on the elution pattern of Factor VIII in 0.25 M $CaCl_2$ was examined. Although these studies were performed under identical conditions with a constant sample volume of 0.4 ml on the same carefully calibrated column, the elution position of the activity peak varied with protein concentration. As shown in Fig. 4, when 0.4 absorbance U

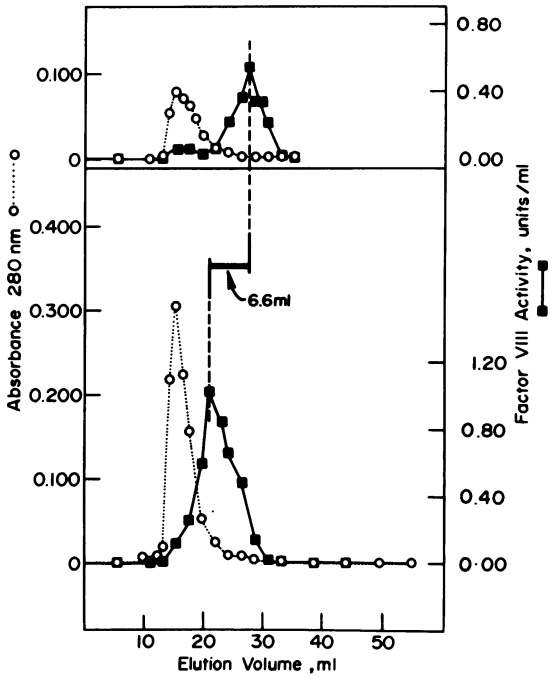


FIGURE 4 4% agarose chromatography of highly purified Factor VIII in 0.25 M $CaCl_2$ (column size: 0.9 × 56 cm) at two different protein concentrations. In both cases the protein peak eluted in the void volume and the PC activity peak eluted later. At the lower protein concentration shown in the upper panel, the PC activity eluted significantly later when compared to the higher protein concentration shown in the lower panel.

was applied, the PC activity peak eluted at a volume of 27.5 ml; however, when 1.5 absorbance U were applied, the PC activity peak eluted at 20.9 ml, a difference of 6.6 ml. Usually the void volume protein peaks in 0.25 M $CaCl_2$ or 1.0 M NaCl were somewhat broader than those observed in 0.15 M NaCl; however, the broadening could not be related to the amount of protein applied to the column. Fig. 5 presents additional data showing that the elution volume of the PC activity peak varied as a function of the protein concentration applied. In general, the peak eluted later when lower protein concentrations were applied. For a dilute protein solution, the activity peak eluted close to the inner volume of the column. The large variation in elution position of the PC activity peak with the protein concentration applied indicates that the material responsible for the activity peak was not filtering strictly according to size or shape. Hence, interaction with the agarose might be occurring or the material responsible for the PC activity might be a concentration-dependent associating system.

Chromatography of Factor VIII in 0.25 M $CaCl_2$ on Sephadex G-200. Though varied, the elution positions of the PC activity peak from 4% agarose in 0.25 M $CaCl_2$ suggested that it has a molecular weight of 200,000 daltons or less. Therefore, the activity peak material would be expected to enter a Sephadex G-200 column, which has an exclusion limit of about 400,000 daltons. Yet as shown in Fig. 6, when 0.15 absorbance U of Factor VIII was rechromatographed in 0.25 M $CaCl_2$ on a 0.9 × 12-cm or 0.9 × 26-cm Sephadex G-200 column, protein and PC activity eluted together as a sharp peak in the void volume.

Effect of 0.25 M $CaCl_2$ on Factor VIII PC activity. Dissociation of Factor VIII into a small active subunit and a carrier protein in 0.25 M $CaCl_2$, would be expected to affect activity in one of three possible ways: (*a*) no change in activity; (*b*) an increase in activity, particularly if dissociation were prerequisite for manifestation of activity; or (*c*) a decrease in activity, especially if the small active subunit were more stable when bound to the carrier protein. To date, it has been suggested that the first situation prevails, i.e., that the small active subunit retains about 80–90% of its activity when dissociated from the carrier protein (10). This question was reexamined in the present study by assaying 20 concentrations of Factor VIII in 0.15 M NaCl and then in 0.25 M $CaCl_2$. Before assay, the sample in 0.15 M NaCl and the sample in 0.25 M $CaCl_2$ were both diluted and adjusted to give 0.043 M $CaCl_2$-0.05 M Tris-0.15 M NaCl. For serial dilutions of both samples, 0.15 M NaCl-0.05 M Tris-0.043 M $CaCl_2$ was used. Both samples were then assayed using the modifications described in the Methods section for samples containing $CaCl_2$ and referred to the

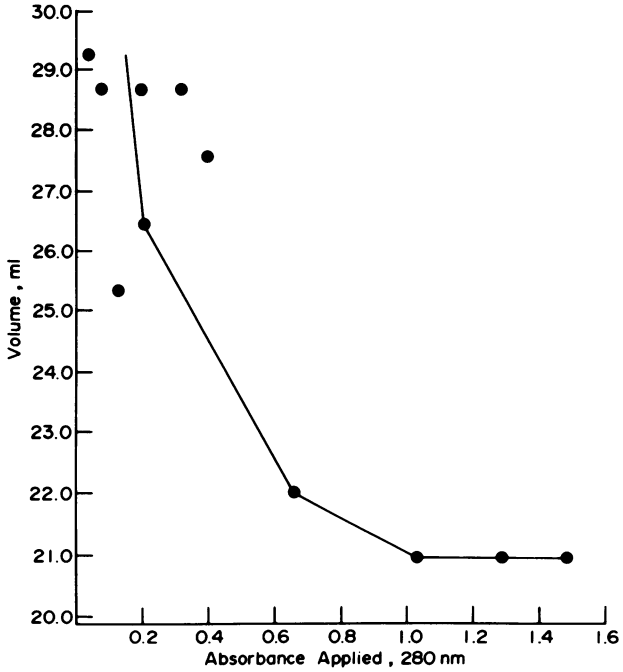


FIGURE 5 Elution volume of the PC activity peak from 4% agarose in 0.25 M $CaCl_2$ as a function of the protein concentration (absorbance at 280 nm) applied (column size: 0.9 × 56 cm).

same reference curve. In both 0.15 M NaCl and 0.25 M $CaCl_2$, Factor VIII specific activity increased as the concentration of Factor VIII protein decreased. This observation cannot be explained by dissociation, since dissociation is reported to occur only in 0.25 M $CaCl_2$ but not in 0.15 M NaCl (10–14). It should also be noted that, in general, incubation of purified Factor VIII with 0.25 M $CaCl_2$ resulted in an immediate loss of PC activity with respect to its paired control in 0.15 M NaCl. Furthermore, this loss of activity in 0.25 M $CaCl_2$ was more pronounced at lower Factor VIII concentrations. Fig. 7 shows that although Factor VIII specific activity in 0.25 M $CaCl_2$ increased as protein concentration decreased, the increase in specific activity was still less than that observed when compared to the samples in 0.15 M NaCl.

Assays for protein, phospholipid, and polyethylene glycol. After concentration by lyophilization and reconstitution in small volumes of buffer, the total protein recovered in the PC activity peak from 4% agarose in 0.25 M $CaCl_2$ was usually about 1% of the applied protein when determined by absorbance at 280 nm or by the Lowry method. Although not quantitated rigorously, it was our impression that larger columns gave poorer yields of the activity peak protein. The ultraviolet spectrum of the lyophilized, reconstituted activity peak material was typical for a protein, with a broad shoulder at about 270 nm and a strong peak below 200 nm.

Factor VIII contained no measurable phosphate by the ascorbic-molybdate method, which should have detected 1 mole or more of phosphate per mole of Factor VIII. Hence, it is reasonable to exclude the possibility that the shifted PC activity peak is related to the separation of noncovalently-bound phospholipid alone or a phospholipid-rich Factor VIII complex.

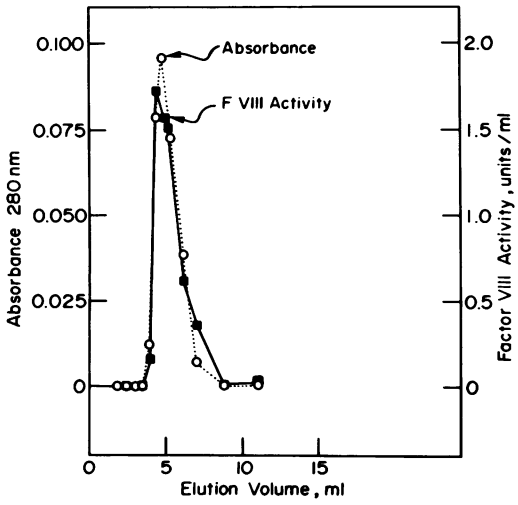


FIGURE 6 Sephadex G-200 gel filtration of highly purified Factor VIII in 0.25 M $CaCl_2$ (column size: 0.9 × 12 cm; identical results were obtained on a 0.9 × 26-cm column). Protein and PC activity were eluted in the void volume.

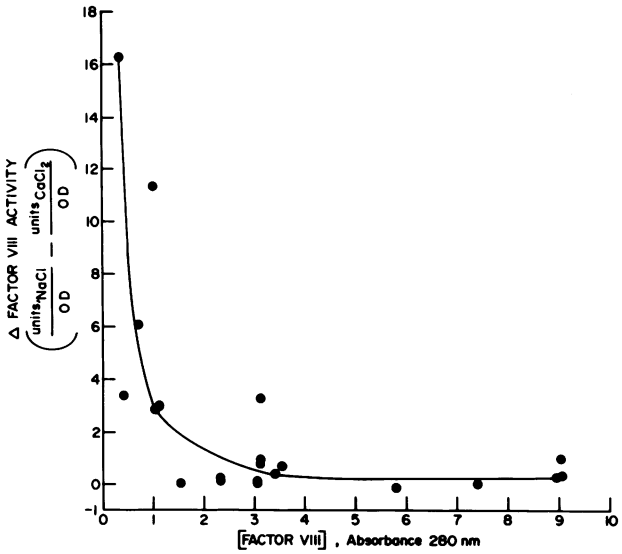


FIGURE 7 Effect of 0.25 M $CaCl_2$ on Factor VIII PC activity. The increase in the specific activity of Factor VIII in 0.15 M NaCl compared to its specific activity in 0.25 M $CaCl_2$ is expressed as a function of protein concentration. The activity of Factor VIII in 0.15 M NaCl was greater than the activity of the same sample in $CaCl_2$ at all protein concentrations. This difference was particularly striking at low protein concentrations.

The possibility that polyethylene glycol, alone or in concert with trace Factor VIII or other clotting factors, could account for the shifted peak of PC activity in 0.25 M $CaCl_2$ was discounted since Factor VIII purified without using polyethylene glycol still gave a shifted activity peak. In addition, aliquots of fractions from the chromatography of polyethylene glycol alone in 0.25 M $CaCl_2$ had no effect on the clotting time of the Factor VIII assay.

Assays of other clotting factors. After concentration and reconstitution to an absorbance at 280 nm of 0.20, the PC activity peak was tested for other clotting factors. The activity peak protein gave clotting times identical to buffer controls in specific assays for Factors IX, X, and Xa. Furthermore, no thrombin activity was detected in an assay system which was sensitive to as little as 0.2 NIH U/ml. Similar results were obtained when selected activity peak fractions were examined before concentration. Hence, the PC activity resolved by agarose gel filtration in 0.25 M $CaCl_2$ is not due to clotting factors which might mimic Factor VIII activity.

Comparison of native Factor VIII to the void volume protein and the activity peak protein. While others have reported that the activity peak protein has PC but not vWF activity (13, 14), in our experiments it had both

TABLE I

*Relative Specific PC and vWF Activities of Native Factor VIII and Pools from $CaCl_2$-4% Agarose Columns**

	Relative PC activity	Relative vWF activity
Native Factor VIII in 0.15 M NaCl	1	1
Native Factor VIII in 0.25 M $CaCl_2$	0.70	0.36
$CaCl_2$ void volume protein	0.03–0.17‡	0.37
$CaCl_2$ activity peak	2–55‡	0.29
lyophilized and reconstituted	0.37	0.15

* The values are averages of at least five separate experiments and normalized to the specific PC and specific vWF activities of native Factor VIII in 0.15 M NaCl, by using absorbance at 280 nm to approximate protein concentrations.

‡ These values varied considerably depending on the resolution of the PC activity peak from the void volume protein; hence, a range seemed more meaningful than a mean value.

activities. The possibility that the observed vWF activity resulted from "tailing" of void volume protein into the PC activity peak region is not likely, since the activity peak protein, unlike either the void volume protein or native Factor VIII, was very soluble in water (2 absorbance U at 280 nm). Table I compares the relative specific PC and vWF activities of native Factor VIII to those of the void volume protein and activity peak protein. The void volume protein had only about 3-17% of the specific PC activity of native Factor VIII. The PC activity peak

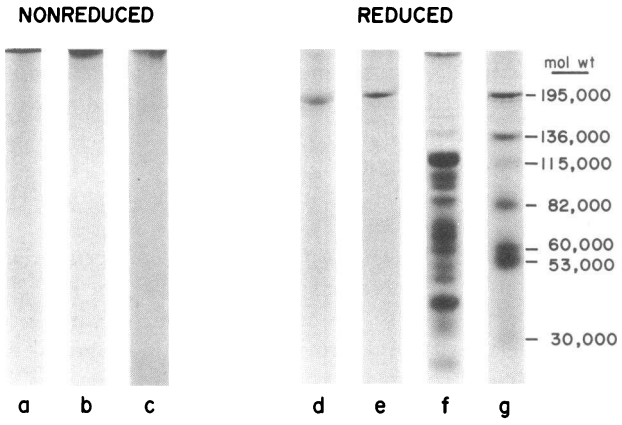


FIGURE 8 SDS 5% gel electrophoresis of: (*a*) nonreduced native Factor VIII; (*b*) nonreduced void volume protein from the 0.25 M $CaCl_2$-agarose columns; (*c*) nonreduced activity peak protein from the 0.25 M $CaCl_2$-agarose columns; (*d*) reduced native Factor VIII; (*e*) reduced void volume protein from the 0.25 M $CaCl_2$-agarose column; (*f*) reduced plasmin-digested Factor VIII; (*g*) reduced activity peak protein from the 0.25 M $CaCl_2$-agarose column. Reduced gels *d*, *e*, and *g* were run at different protein loads and the patterns normalized to the intensity of the 195,000 dalton band so that the relative amounts of additional subunit bands as seen on gel *g* could be easily appreciated.

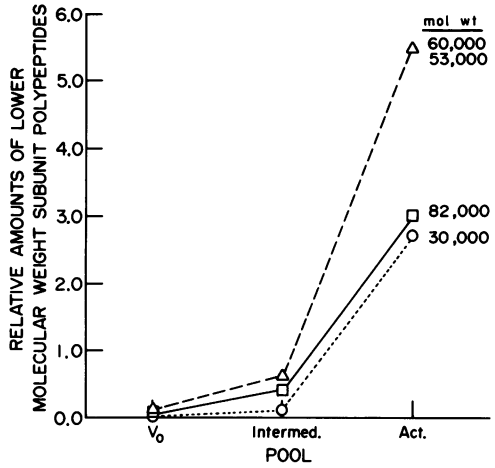


FIGURE 9 Relative intensities of the most prominent subunit polypeptides as analyzed by SDS-gel electrophoresis after reduction of protein in each of the three pools from $CaCl_2$-agarose chromatography of Factor VIII. (*a*) Void volume; (*b*) intermediate zone between void volume and activity peak; (*c*) activity peak protein. The intensity of each band was normalized to the intensity of the 195,000-dalton subunit observed in each of the three patterns. Clearly the lower molecular weight subunit polypeptides peaked in the PC activity region of the chromatogram.

protein, however, had a specific Factor VIII activity much greater than that of native Factor VIII and retained some PC activity even after dialysis, lyophilization, and reconstitution.

The specific vWF activity was computed from the ristocetin-induced platelet aggregation data as described in the Methods section. The PC activity peak protein from the 0.25 M $CaCl_2$-agarose columns had slightly less specific vWF activity than the void volume protein, although both had only ~30% of the vWF activity of native Factor VIII in 0.15 M NaCl. The observed decrease in vWF activity in $CaCl_2$ is probably a calcium ion effect, since native Factor VIII made 0.25 M in $CaCl_2$ had only about 36% of the specific vWF activity of native Factor VIII in 0.15 M NaCl. The similarity of the specific vWF activities of both the void volume and activity peak proteins to that of native Factor VIII in 0.25 M $CaCl_2$ is evidence that both are forms of Factor VIII. Yet, as indicated in Table I, these forms of Factor VIII are quite different in specific PC activity. As shown in Table I the lyophilized, reconstituted activity peak protein still had platelet-aggregating activity.

In the presence of 0.25 M $CaCl_2$, native Factor VIII as well as the void volume and the PC activity peak samples from a 0.25 M $CaCl_2$-agarose column lost about 88% of their PC activity immediately upon the addition of adsorbed human plasma which contained a high titer of Factor VIII antibody. Both the void volume sample (PC activity of 0.37 U/ml) and the activity peak sample (PC activity of 0.68 U/ml) were completely inactivated

within 1 min. Native Factor VIII (PC activity of 1.60 U/ml) lost 88% of its activity after incubating for 1 min with the antibody and 99% of its activity after 72 min.

SDS-gel electrophoresis of the activity peak protein. To date, no structural information has been reported for the activity peak protein. Fig. 8 shows the SDS-5% gel electrophoretic patterns for native Factor VIII, plasmin-digested Factor VIII, and the void volume protein and activity peak protein recovered from the $CaCl_2$-agarose column. The nonreduced SDS-gel patterns of the void volume protein and PC activity peak protein were identical to that of native Factor VIII, all showed protein that did not enter a SDS-5% polyacrylamide gel. Fig. 8 also shows the SDS-gel electrophoretic patterns for the same protein samples, but after reduction by β-mercaptoethanol. Both native Factor VIII and the void volume protein were reduced to a single 195,000-dalton peptide. The activity peak protein, however, was reduced into five major subunit polypeptides which had molecular weights of 195,000, 136,000, 82,000, 60,000, and 53,000 daltons. Two minor bands with molecular weights of 115,000 and 30,000 daltons were also present. SDS-gel analyses of several different preparations of the PC activity peak protein showed that the bands of 82,000, 60,000, and 53,000 daltons were the most prominent. As shown in Fig. 9, when SDS gels of *reduced* samples with the same protein concentration from different areas of the elution pattern were analyzed, the greatest intensity of the multiple polypeptide band pattern (gel *g*, Fig. 8) did in fact coincide with the activity peak. Fractions subsequent to the activity peak did not contain protein. While the reduced gel of plasmin-treated Factor VIII is clearly different from that of the activity peak protein, there are enough qualitative similarities to suggest that the SDS-gel pattern of the reduced PC activity peak protein also results from proteolytic cleavage of the 195,000-dalton subunit of native Factor VIII. As a control, fractions from the region of the 4% agarose-0.15 M NaCl chromatogram of Factor VIII that corresponded to the activity peak region from the 4% agarose-0.25 M $CaCl_2$ column were also concentrated and analyzed by SDS-gel electrophoresis. At best a trace band could be seen occasionally in the 80,000- to 60,000-dalton region after reduction. Unlike elutions from the agarose-$CaCl_2$ columns, however, usually no protein or activity was recovered from this area of the chromatogram when 0.15 M NaCl was used as the eluent.

DISCUSSION

The central issue in the current controversy about the molecular structure of Factor VIII is whether the molecule has a covalent structure and contains both PC and vWF activities or whether the molecule is composed of two noncovalently joined subunits, one of which is small and contains the PC activity and the other of which is large and contains the vWF activity. Obviously the controversy is not a moot point since each model has important and quite different implications to the genetics as well as to the future therapy of both hemophilia and von Willebrand's disease.

In the present study, we were unable to confirm recent reports (9, 11, 14) that Factor VIII dissociates when filtered through 4% agarose in 0.80–1.0 M NaCl. Instead, the protein, PC, and vWF activities eluted together in the void volume just as they did in 0.15 M NaCl. Admittedly, highly purified Factor VIII was used in our studies while only partially purified Factor VIII or crude concentrates were used in those studies where dissociation was observed (9, 11, 14). While perhaps it is possible that some change in the Factor VIII molecule occurred during its purification that prevented dissociation, this explanation is not likely, since SDS-gel analyses showed only the expected bands before or after reduction. In view of the property of 1% SDS-5 M urea to dissociate the noncovalent structure of a large spectrum of proteins, the active subunit, reportedly around 100,000 daltons in molecular weight, should have been apparent as a band within a SDS 5% gel, provided that it was present in high enough concentrations and that it stained with Coomassie blue.

When purified Factor VIII was rechromatographed on 4% agarose in 0.25 M $CaCl_2$, a protein peak similar to that observed on the NaCl columns eluted in the void volume and as others have noted (9–16), the peak of PC activity eluted later. Contrary to expectations, the elution position of the PC activity peak was very sensitive to the protein concentration of the applied sample, despite the use of identical conditions and the same column. Figs. 4 and 5 clearly demonstrate that the PC activity peak was included farther into the column as the protein concentration of the applied sample decreased. Such variation has not been mentioned in reports by other investigators who have observed the "dissociation" of PC activity from the void volume protein in the presence of 0.25 M $CaCl_2$. Probably our observations account for the widely different molecular weights for the PC activity peak protein reported by the same investigators (10, 12) as well as by different investigators (9). A protein or peptide of constant molecular weight should elute at the same position when identical chromatographic conditions are employed unless: (*a*) complete dissociation to a smaller species occurs only at low protein concentrations, above which monomeric forms become bound to each other or to a carrier protein; or (*b*) the elution position of the PC activity peak is not simply a function of molecular size, but instead results from varying degrees of adsorption to the agarose gel.

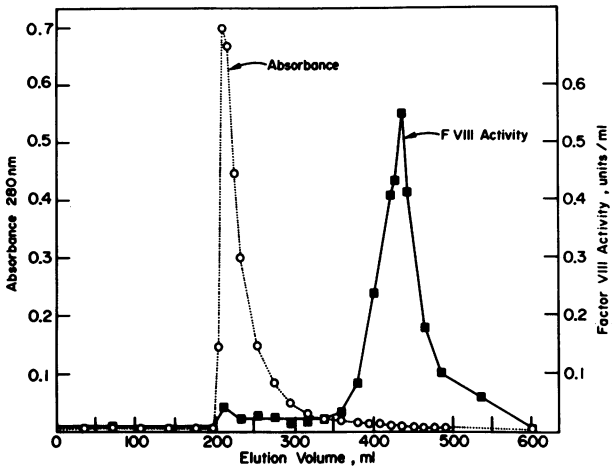


FIGURE 10 Gel filtration of highly purified Factor VIII on 4% agarose in 0.25 M $CaCl_2$ (column size: 2.5 × 98 cm). This is the best resolution of the PC activity peak from the void volume protein; however, this degree of resolution was not reproducible. Furthermore, exceedingly low yields of PC activity peak protein were recovered from this size column, presumably due to adsorption to the agarose in the presence of 0.25 M $CaCl_2$.

Several considerations appear to discount the first possibility. Data from the present study as well as from others (10–12, 14) suggest that at least 70% of the PC activity becomes dissociated from the void volume protein in 0.25 M $CaCl_2$. Since all investigators agree that the carrier protein is essentially excluded by the agarose, any undissociated carrier protein-active subunit complex would be eluted in the void volume. If the PC activity peak shifted farther into the column because of increased dissociation of the active subunit from the carrier protein, one would expect the completely dissociated active subunit to be eluted at a volume at least as great as that observed for the most dilute protein solution applied. For the examples given in Figs. 4 and 5, this volume is 27.5 ml while the void volume for the same column is 15.4 ml. With such great separation between the void volume and the elution volume of the PC activity peak, a peak of PC activity corresponding to the undissociated carrier protein-active subunit complex should elute in the void volume, and a second peak of PC activity corresponding to the fully dissociated subunit should elute much later. Hence, the observation of only a single, symmetrical activity peak is not consistent with the explanation of increasing dissociation of the active subunit from the carrier protein at low protein concentrations. If, however, the active subunit has a molecular weight of 100,000–200,000 daltons and aggregates with itself, an aggregate of six or more active subunits might be expected to elute in the void volume. Smaller aggregates would then be expected to elute after the void volume. Assuming that the size of the aggregate is strictly a function of protein concentration, one would expect to produce a distribution of aggregate sizes at each concentration, with the smaller aggregates eluting later from the column. A single peak of activity that shifts on dilution of the applied sample is therefore consistent with the formation of smaller aggregates of active subunits. Our Sephadex G-200 chromatography data, however, discount this hypothesis that the varying elution position of the PC activity peak results from varying stages of dissociation or aggregation and instead support the second hypothesis that different degrees of adsorption lead to the varying elution positions. If the PC activity peak protein were as small as proposed by other investigators (10, 12), it should also dissociate from the void volume protein on Sephadex G-200, which has an exclusion limit of about 400,000 daltons. However, when purified Factor VIII was rechromatographed on Sephadex G-200 in 0.25 M $CaCl_2$, both protein and PC activity always eluted together in the void volume. The observations that the PC activity peak was eluted in the void volume on Sephadex G-200 and at varying volumes on 4% agarose suggest that the PC activity peak protein is large and that adsorption to the agarose in the presence of 0.25 M $CaCl_2$ is occurring to some extent. Our results, however, cannot be reconciled with the report of others (12) that a small active subunit is included in Sephadex G-150 in 0.25 M $CaCl_2$.

To date, the stoichiometry of the large carrier protein and active subunit complex has not been addressed by any investigators, despite the finding of little or no protein beneath the displaced PC activity peak separated on agarose in 0.25 M $CaCl_2$. Fig. 10, a chromatogram of purified Factor VIII filtered through 4% agarose in 0.25 M $CaCl_2$, shows that a protein peak with a maximum absorbance of 0.700 U eluted sharply in the void volume the PC activity peak eluted much later and had a flat absorbance of ~0.001 U. If it is assumed that the absorbance at 280 or 210 nm is proportional to the protein concentration, then the weight ratio of carrier protein to active subunit is 700:1. Assuming a molecular weight of about 10^6 daltons for the carrier protein and a 1:1 mole ratio of carrier protein to active subunit would yield a molecular weight estimate of 1,500 daltons for the active subunit. Even the assumption of widely divergent extinction coefficients for the carrier protein and active subunit does not account for any reasonable stoichiometry compatible with current experimental observations. This estimate is much smaller than the 100,000 daltons suggested by other investigators (12). An alternative explanation for the very small amount of protein under the activity peak is that many molecules of carrier protein are associated with only one molecule of active subunit. To account for an active subunit as large as 100,000 daltons, 70 carrier protein molecules per active subunit

would be required. Such an occurrence seems unlikely from a steric standpoint alone; in addition the molecular weight of such a complex would approximate 70 million daltons.

To date, the fact that the standard assay of Factor VIII PC activity is not straightforward in the presence of 0.25 M $CaCl_2$ has received little or no attention. The results of the present study show that about 30% of the PC activity is lost immediately in 0.25 M $CaCl_2$; furthermore, if the PC activity in 0.25 M $CaCl_2$ is adjusted back to that in 0.15 M NaCl, the recovery of PC activity from agarose chromatography in 0.25 M $CaCl_2$ approximates only about 55%. Our observation that the specific activity of Factor VIII increases on dilution in 0.15 M NaCl, but less so in 0.25 M $CaCl_2$ obviates a specific promotion of dissociation by 0.25 M $CaCl_2$ (10–12).

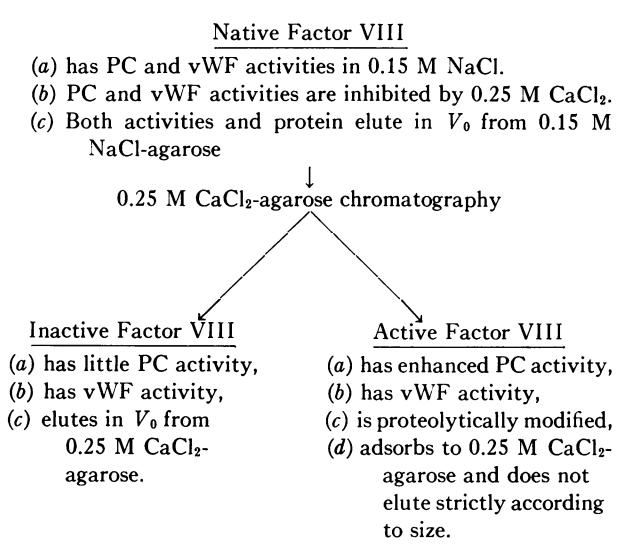
Isolation and characterization of the PC activity peak material from agarose chromatography in 0.25 M $CaCl_2$ indicate that it is protein. The results of specific assays for phospholipid, Factors IX, X, and Xa and thrombin were negative. The PC activity peak protein does, however, possess many properties heretofore associated with Factor VIII. It has both PC and vWF activities and furthermore the PC activity is totally abolished by a human Factor VIII antibody. The observation that, unlike the void volume protein, it is very soluble in water (~ 2 absorbance U at 280 nm) discounts the possibility that the PC activity peak protein is merely void volume protein that "tails" into the PC activity peak.

Measurement of the vWF activity is a sensitive method of detecting native or modified Factor VIII. Native Factor VIII, thrombin-inactivated Factor VIII, and Factor VIII inactivated by a human antibody all retain essentially full vWF activity (27). Even after 24 h of plasmin digestion, which results in degradation of its subunit structure, Factor VIII still retains $\sim 80\%$ of its vWF activity. Hence, as shown in Table I, the similarity of the specific vWF activities of the void volume and activity peak proteins to that of native Factor VIII in 0.25 M $CaCl_2$ suggests that both are native or modified forms of Factor VIII. Factor VIII PC activity is extremely sensitive (27) to activation or inactivation by a variety of chemical or enzymatic means. Thus, the large differences among the specific PC activities of native Factor VIII in $CaCl_2$, the void volume protein, and the activity peak protein are not surprising. The void volume protein appears to be a nonactive form of Factor VIII, possibly inactivated by Ca^{++}. The activity peak protein has a higher specific activity than native Factor VIII in $CaCl_2$, possibly because of activation by a protease. Therefore, the different PC/vWF ratios for native Factor VIII in $CaCl_2$, the void volume protein, and the activity peak protein reflect only the respective differences in the specific PC activities, since the specific vWF activities were about the same for the three cases.

The first structural information on the activity peak protein is provided by our SDS-gel electrophoretic data. Like native Factor VIII and the void volume protein, the activity peak protein contains material that does not enter a SDS 5% gel in the absence of reducing reagent. This result indicates that the activity peak protein is large and does not contain noncovalently bonded subunits smaller than $\sim 400{,}000$ daltons. On reduction, the void volume protein and native Factor VIII show identical gel patterns, which contain only the 195,000 dalton subunit; however, the gel pattern for the reduced activity peak protein is different. It shows a series of seven bands, the molecular weights of which range from 195,000 to 30,000 daltons. The higher molecular weight bands of the reduced activity peak protein varied in intensity from one preparation to another but they were always less intense than the lower molecular weight bands of 82,000, 60,000, and 53,000 daltons. The SDS-gel pattern of the reduced activity peak was reminiscent of patterns resulting from plasmin degradation of Factor VIII not exposed to $CaCl_2$. There are, however, major differences between SDS-gel patterns of the reduced activity peak protein and plasmin-degraded Factor VIII. For example, the activity peak protein has more high molecular weight bands and fewer bands overall than plasmin-treated Factor VIII. In addition, treatment of Factor VIII by plasmin in several different ratios always resulted in a loss and never an enhancement of Factor VIII activity. These observations indicate that the SDS-gel pattern for the reduced activity peak protein was not due to degradation of factor VIII by plasmin. Quite possibly thrombin, which is known to enhance Factor VIII PC activity (6, 7, 14, 27), could be the protease involved. For example, a small amount of Factor VIII might be partially activated by thrombin in vivo or during the early stages of plasma processing. Since thrombin-modified Factor VIII subunits would still be covalently joined by disulfide bonds to form a macromolecule with a molecular weight of one million or more, a small amount of thrombin-modified Factor VIII should copurify with native Factor VIII; however, the modifications might be such that the thrombin-activated Factor VIII easily adsorbs to agarose in the presence of 0.25 M $CaCl_2$. This suggestion that the PC activity peak protein represents a modified, partially activated form of Factor VIII concentrated by adsorption onto agarose in 0.25 M $CaCl_2$ does not conflict with reports that thrombin produces no detectable modification of Factor VIII (6, 7). Instead, the earlier reports probably reflect the inability of the SDS-gel technique to detect a modified form of Factor VIII which constitutes less than 1% of purified Factor VIII preparations isolated by agarose chromatography in 0.15 M NaCl. Finally, the report that thrombin can activate the PC ac-

tivity peak protein (29, 30) could be explained by further activation of an already partially modified Factor VIII species.

In conclusion, our results indicate that the PC activity peak of protein is large, > 400,000 daltons, and is composed of several disulfide-bound nonidentical subunits. The activity peak protein does not filter true to size in that it appears to interact with the agarose gel in 0.25 M $CaCl_2$. It is suggested that the PC activity peak protein is a modified form of native Factor VIII on the basis of its apparent size when nonreduced, its multiple band pattern after reduction, and its possession of vWF activity as well as PC activity. Our data from the $CaCl_2$ studies, suggest that only a very small proportion of circulating Factor VIII protein is active, possibly as a result of thrombin action, whereas the remainder has perhaps been inactivated by certain proteolytic enzymes, or remains in a precursive state, or is inhibited directly by 0.25 M $CaCl_2$. The following schematic depicts the interpretation of our data.



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