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Research Article

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Studies on the Purification and Characterization of Human Factor VIII

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ABSTRACT Factor VIII (antihemophilic globulin) has been prepared from Hyland method IV AHG and cryoprecipitate using limited chymotryptic digestion followed by Sepharose gel filtration. The activity of factor VIII is unaffected by the digestion procedure, while fibrinogen is converted to large noncoagulable fragments.

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INTRODUCTION

The first 10 years of work on the purification of antihemophilic factor, begun by the Blombäcks in the mid-1950's, produced factor VIII concentrates which have greatly improved the clinical management of hemophilia. More recently, attention has been turned toward purification and analysis of the factor VIII molecule. With the development of approaches and techniques for characterizing normal factor VIII, it may be possible to define the molecular abnormalities of hemophilia and von Willebrand's disease.

The purification of factor VIII from plasma has been a difficult problem for three major reasons. (*a*) The low concentration (probably < 10 μg/ml) of factor VIII protein in plasma; (*b*) instability of the procoagulant activity; and (*c*) the difficulty encountered experimentally in separating fibrinogen from factor VIII.

Factor VIII can be concentrated from plasma by precipitation with a variety of agents, including ethanol (1, 2) amino acids (3, 4) or amino acid analogues (4, 5), polyethylene glycol (6), or by cryoprecipitation (7). These agents precipitate large amounts of fibrinogen in addition to factor VIII activity. Partial removal of fibrinogen can be achieved by adsorption with bentonite or fuller's earth (5, 6, 8), but this step may also remove significant amounts of factor VIII activity.

Since the development of large pore gel filtration for the separation of macromolecules, filtration on agarose has been widely used as a final step in the purification of factor VIII (8–12). In this step, factor VIII is recovered in or near the exclusion volume, suggesting a molecular weight greater than 2×10^6 (8, 13, 14).

The increase in the specific activity of factor VIII achieved by these procedures is in the range of 3000–10,000 when compared with plasma (6, 10, 11). However, it must be emphasized that the degree of purity of these preparations has not been established. Analysis of highly purified factor VIII preparations by electrophoresis on polyacrylamide gels has been inconclusive, since the material does not enter the gel.

Several investigators have suggested that fibrinogen may be separated from factor VIII by digestion with snake venoms (15). In preliminary studies in our laboratory, digestion of fibrinogen in Cohn fraction I by *Crotalus terrificus* venom was accompanied by significant loss of factor VIII activity. Digestion by other

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proteolytic enzymes, such as plasmin and trypsin, was also accompanied by large losses of factor VIII activity. However, it was found that low concentrations of α-chymotrypsin will digest fibrinogen into noncoagulable fragments with complete sparing of factor VIII activity. The present report describes a method for the rapid separation of factor VIII from fibrinogen by mild chymotryptic digestion of crude factor VIII concentrates, followed by Sepharose gel filtration.

The purified factor VIII preparation has been analyzed chemically for sugars, lipids, and amino acids, and the subunit structure has been studied by polyacrylamide gel electrophoresis. Crude and purified factor VIII has also been studied by immunoelectrophoresis, using an antibody made in rabbits to the purified factor VIII preparation.

The method to be described can be used to prepare factor VIII in quantities sufficient for analysis from 2 U of plasma. Thus, it is a useful method for studying the plasma of individual patients with hemophilia or von Willebrand's disease.

METHODS

Crude factor VIII concentrates. Method IV AHG, a factor VIII concentrate prepared from human plasma, was kindly supplied by Hyland Div., Travenol Labs, Costa Mesa, Calif. *Cryoprecipitate* was provided by the Blood Bank of the National Institutes of Health. It was prepared by thawing fresh frozen plasma at 4°C overnight and subsequent removal of supernatant plasma.

α-Chymotrypsin from bovine pancreas was obtained from Worthington Biochemical Corp., Freehold, N. J. The chymotrypsin was freshly dissolved for each experiment in 0.001 N HCl at concentrations of 1–10 mg/ml.

Diphenylcarbamyl chloride (DPCC)[1] was obtained from Mann Research Laboratories, Inc., New York.

Fibrinogen was purified from human plasma by repeated precipitation with ammonium sulfate at 25% saturation. The final product was 92% coagulable.

Coagulable protein. The fibrinogen content of Hyland method IV and cryoprecipitate was estimated by measurement of coagulable protein. The solution was diluted to an OD_{280} of 1.0, bovine thrombin (Parke, Davis & Company, Detroit, Mich.) was added at a final concentration of 10 U/ml, and the clot formed was removed after a 90 min incubation at room temperature. Coagulable protein was estimated by subtracting OD_{280} of supernate after clotting from OD_{280} of starting solution.

Coagulation assays. All coagulation tests were performed on a Fibrometer (BioQuest Div., Cockeysville, Md.). The one-stage factor VIII assay used was a partial thromboplastin time with hemophilic plasma as substrate (16). The two-stage factor VIII assay used was the modified thromboplastin generation test described by Pool and Robinson (17). Thrombin clotting times were performed by adding 0.1 ml substrate containing fibrinogen to 0.2 ml bovine thrombin solution (Parke, Davis & Company) at 37°C and measuring the time for clotting to occur. The thrombin time was 25–30 sec using a thrombin concentration of 2–3 U/ml (for clotting Hyland method IV) or 0.25 U/ml (for clotting cryoprecipitate).

Gel filtration was performed on a 5 × 100 cm Sephadex column packed with Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). The void volume was determined with dextran blue 2000 (Pharmacia). Columns were eluted at 4°C with buffer containing 0.05 M Tris (Mann Ultrapure, Mann Research Laboratories, Inc., New York) and 0.1 M NaCl, adjusted with HCl to pH 7.4. The factor VIII eluate from Sepharose gel filtration was concentrated up to 250 times by dialysis against powdered Ficoll. For analytic studies, samples were then dialyzed against distilled water and lyophilized in a VirTis Freeze-Mobil (VirTis Co., Gardiner, N. Y.).

Preparation of antibody to factor VIII. Antibody was developed in New Zealand rabbits to purified factor VIII prepared as described in the Results. Rabbits were injected subcutaneously on days 1 and 10 with 1 mg of purified lyophilized factor VIII protein (Table I) suspended in incomplete Freund's adjuvant (Difco Labs, Detroit, Mich.). On days 10, 20, and 30 the rabbits were bled by heart puncture. The blood was allowed to clot at 37°C for 4 hr, the serum was separated by centrifugation at 3000 rpm for 20 min and stored at −60°C. Absorption of antiserum with normal or hemophilic plasma (ratio 3 parts antiserum to 1 part plasma) was performed at 37°C for 1 hr followed by 12 hr at 4°C. Supernatant serum was removed after centrifugation at 30,000 rpm for 30 min.

Immunodiffusion on Hyland agar gel immunoplates was used to detect fibrinogen in purified factor VIII preparations. Samples were run against antihuman fibrinogen prepared in rabbits, and the sensitivity of the method was checked with serial dilutions of purified fibrinogen.

Immunoelectrophoresis was performed on 1.5% Ionagar plates (3½ × 4 inches) prepared in 0.05 M veronal buffer pH 8.6. Antigen (10 μl crude or purified factor VIII) was electrophoresed in the same buffer for 2 hr at 40 mv. The purified factor VIII antigen used was the "factor VIII pool" from gel filtration (see Fig. 2) concentrated 10 times. After electrophoresis, the troughs were filled with approximately 200 μl rabbit antifactor VIII serum and diffusion was allowed to proceed for 40 hr at room temperature in a moisture chamber. The slides were stained with Amidoschwartz.

Acrylamide gel electrophoresis was performed using the reagents and methods described by Canalco Inc., Rockville, Md. Standard 5% acrylamide gels were run for 3–22 hr in 0.05 M Tris − 0.38 M glycine, pH 8.4 at 4°C. Electrophoresis was also performed in 5% acrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (Pierce Chemical Co., Rockford, Ill.) according to Shapiro, Viñuela, and Maizel (18). Samples for electrophoresis in SDS-acrylamide gels were first incubated for 3 hr at 37°C in 1% SDS-0.1 M sodium phosphate buffer (pH 7.1) containing 0.025% sodium azide with or without the addition of the disulfide-reducing agent dithioerythritol (Mann Research Laboratories, Inc.). Electrophoresis was carried out for 3 hr at room temperature in 0.1% SDS-0.1 M sodium phosphate buffer, pH 7.1. The gels were stained with coomassie blue after fixation overnight in 20% sulfosalicylic acid, or with periodic acid-Schiff (PAS) after fixation in 5% acetic acid-40% ethanol.

The SDS-acrylamide gel system was used as a means of estimating molecular weight. Proteins of known molecular

[1] *Abbreviations used in this paper:* DPCC, diphenylcarbamyl chloride; PAS, periodic acid-Schiff; SDS, sodium dodecyl sulfate.

weight were incubated in SDS and electrophoresed on SDS-acrylamide gels as described above, and a linear plot was drawn relating centimeters migrated in the gel to log of molecular weight (18). The migration on the gels of unknown samples was measured and approximate molecular weights obtained. Proteins used as standards included ovalbumin (43,000 mol wt), bovine serum albumin (68,000 mol wt), transferrin (90,000 mol wt) and γ-globulin (160,000 mol wt unreduced; 55,000 and 23,000 after disulfide reduction). The migration of fibrinogen (330,000 mol wt) was also used for high molecular weight estimations.

Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (19).

Sialic acid was determined on 0.1 N HCl hydrolysates of lyophilized samples by the method of Warren (20). *N*-acetyl-neuraminic acid (Sigma Chemical Co., St. Louis, Mo.) was used as a standard.

Hexoses were determined by paper chromatography in pyridine: ethyl acetate: H_2O, 5:12:4. Before chromatography, the samples were hydrolyzed in 2 N HCl at 100°C for 2 hr, the hydrolysate was passed through a mixed bed resin containing AG 3 × 4 (OH^-) and AG 50W (H^+) (Bio-Rad Labs, Richmond, Calif.) and evaporated to dryness under pressure. Standard hexose solutions containing glucose, galactose, mannose, fucose, and rhamnose were chromatographed simultaneously. Chromatograms were stained according to Anet and Reynolds (21).

Amino acid analysis. Lyophilized samples were suspended in 6 N HCl, evacuated and sealed, and hydrolyzed for 20 hr at 110°C. The hydrolysate was analyzed on a Hitachi KLA-3B (Hitachi Ltd., Tokyo, Japan) for amino acids and amino sugars.

Lipid analysis. Chloroform-methanol extracts of lyophilized samples were analyzed by thin-layer chromatography on silica gel-coated plates (Adsorbosil-5 Prekotes) (Applied Science Labs, Inc., State College, Pa.). A variety of solvents was used including chloroform-methanol-H_2O 65:25:4; petroleum ether-diethyl ether-acetic acid 90:10:1 or 70:20:4; isopropyl ether-acetic acid 96:4 followed by petroleum ether-diethyl ether-acetic acid 90:10:4. Spots were visualized by charring with H_2SO_4 or 5% phosphomolybdic acid in ethanol. Lipids used as standards included cholesterol, cholesterol esters, methyl oleate, oleic acid, monoolein, triolein, phosphoethanolamine, lecithin, lysolecithin, and sphingomyelin.

RESULTS

The starting material used for the preparation of purified factor VIII was either Hyland method IV AHG or cryoprecipitate. Hyland method IV AHG, which is prepared from human cryoprecipitate by polyethylene glycol precipitation of fibrinogen followed by glycine precipitation of AHG,[2] was used for most of the work to be described. It contains 0.6–0.7 g protein/g lyophilized powder, of which 70% is coagulable protein. The specific activity of factor VIII is Hyland method IV as measured in our laboratory by a one-stage assay, ranges from 0.2 to 0.4 U/mg protein, compared with a specific activity in plasma of 0.014–0.017 U/mg protein. The cryoprecipitate supplied by the National Institutes of Health Blood Bank contains approximately 30–50% coagulable protein, and has a specific activity of 0.06 factor VIII U/mg protein.

Digestion by α-chymotrypsin of factor VIII concentrates. Fig. 1*a* illustrates the chymotryptic digestion of fibrinogen in Hyland method IV AHG. The digestion process was monitored by the increase in thrombin clotting time of the substrate fibrinogen. Factor VIII assay was performed after the thrombin clotting time became > 300 sec and was compared with the factor VIII level of the undigested material. In Fig. 1*a*, the fibrinogen in Hyland method IV AHG became incoagulable at a chymotrypsin concentration of 3 μg/ml or greater. Measurement of factor VIII activity in the noncoagulable chymotrypsin digests showed no loss of activity from an initial level of 8 U/ml. However, in experiments at higher enzyme concentrations than those shown here, at which the fibrinogen became incoagulable in less than 1 hr, factor VIII activity was lost in variable amounts. Because of the possibility that unchanged factor VIII levels during chymotryptic digestion were due to partial loss and partial activation of factor VIII by the enzyme, two-stage factor VIII assays were also performed. Factor VIII levels by one and two-stage methods were comparable in both digested and undigested material, and thus gave no evidence of factor VIII activation.

A similar digestion can be performed on cryoprecipitate, except that in this case much larger amounts of chymotrypsin are required, presumably because of the natural protease inhibitors found in plasma. As shown in Fig. 1*b*, chymotrypsin was added stepwise to cryoprecipitate until an adequate rate of digestion of fibrinogen was obtained. Factor VIII activity in the noncoagulable digest remained at the starting level of 3 U/ml, providing the addition of chymotrypsin was carefully titrated against thrombin clotting time.

In preliminary experiments, attempts were made to stop the chymotryptic digestion with pancreatic trypsin inhibitor (Worthington Biochemical Corp.) (0.6 mg/ml final concentration) and a specific chymotrypsin inhibitor (DPCC at 0.25–0.5 μg/ml). However, because of the instability of chymotrypsin at neutral pH and the low concentrations used, the reaction can be stopped simply by cooling the digest to 4°C and immediately separating the enzyme from factor VIII by gel filtration (see below).

Preparation of purified factor VIII from Hyland method IV AHG. 1½ g of Hyland method IV lyophilized powder, which contains 0.9 g protein, was dissolved in 20 ml distilled water (manufacturer's instructions) and an additional 8 ml of 0.05 M Tris-0.1 M NaCl, pH 7.4, was added. The final pH of this preparation was 7.2, the protein concentration was 30 mg/ml

[2] Personal communication from Hyland Laboratories, Costa Mesa, Calif.

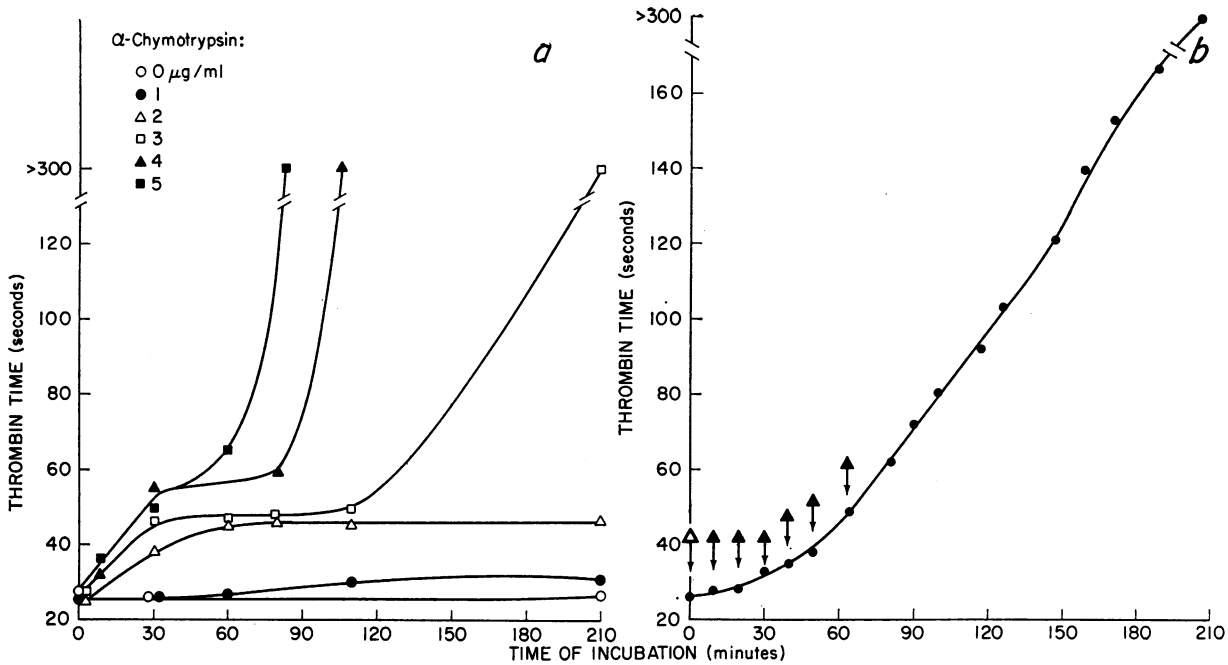

FIGURE 1 (*a*) *Digestion of Hyland method IV AHG by α-chymotrypsin.* Hyland method IV AHG (30 mg protein/ml; factor VIII 8 U/ml) was incubated with 0-5 μg α-chymotrypsin/ml substrate at room temperature. Digestion of fibrinogen was followed by measuring the time for clot formation of 0.1 ml substrate and 0.2 ml bovine thrombin (2 U/ml) at 37°C. (*b*) *Digestion of cryoprecipitate by α-chymotrypsin.* Cryoprecipitate from 2 U of normal human plasma was reconstituted in 24 ml supernatant plasma (50 mg protein/ml, factor VIII 3 U/ml). Chymotrypsin was added at room temperature at 10-min intervals at final concentrations of 125 μg/ml (△) at time zero and at 25 μg/ml (▲) for all subsequent additions. Digestion of fibrinogen was followed by measuring the clotting time of 0.1 ml substrate and 0.2 ml bovine thrombin (0.25 U/ml) at 37°C.

and factor VIII by one-stage assay was 8 U/ml. Digestion of this preparation by chymotrypsin was performed as described above in Fig. 1*a*. The digest was cooled to 4°C, centrifuged at 18,000 rpm to remove small amounts of aggregated protein, and 25 ml was applied to a Sepharose 4B column. A typical elution pattern of factor VIII activity and protein is seen in Fig. 2.

Factor VIII activity begins to emerge at the exclusion volume, indicated by the arrow, and rises in a broad peak to a maximum level of 0.8 U/ml, accompanied by protein levels barely detectable spectrophotometrically. The factor VIII activity is well separated from the bulk of the protein, which is comprised largely of fibrinogen digestion products (see below). This is in contrast to the elution of undigested Hyland method IV in which the factor VIII activity and native fibrinogen overlap broadly.

An important result of the digestion step is that crude factor VIII concentrates containing large amounts of fibrinogen can be filtered through Sepharose at low temperatures. While solutions of Hyland method IV at protein concentrations over 6 mg/ml from a gelatinous aggregate at 4°C, the chymotrypsin-treated material forms a clear solution of low viscosity even at a protein concentration of 30 mg/ml, which is easily filtered through a Sepharose column. Thus, the digestion procedure increases at least fivefold the amount of crude starting material which can be processed at one time. In addition, the digestion procedure markedly improves the separation of factor VIII and fibrinogen on the column.

Column fractions with factor VIII activity and without measurable absorption at 280 mμ ("factor VIII pool" in Fig. 2) were pooled and concentrated 250 times by dialysis against Ficoll powder. The concentrate was then dialyzed against distilled water and lyophilized. Table I compares the stages in the purification scheme with regard to factor VIII activity, protein, and specific activity. The final yield of factor VIII protein from 750 mg starting material was approximately 2.5 mg.

The factor VIII activity in the dilute Sepharose eluate is extremely labile, losing 50% of its activity over-

night at 4°C. Additional activity is lost during concentration of the eluate. Concentration by pressure dialysis was found to cause greater loss of activity, and more visible aggregation of protein, than concentration by dialysis against Ficoll powder, and thus the latter method was used. There was no attempt made to stabilize the factor VIII activity of the Sepharose eluates or concentrates since the material was subsequently lyophilized for analytic studies. However, it was found in pilot experiments that the factor VIII activity of dilute and concentrated column eluates could be preserved at 80–100% of their original levels for as long as 6 days by addition of bovine serum albumin at 10 mg/ml and storage at 4 or −20°C.

Precipitating antibody to factor VIII. Immunoelectrophoresis of purified factor VIII against rabbit anti-factor VIII produced a single precipitin line as shown in Fig. 3*a*. A similar line was also seen using crude factor VIII as antigen (Fig. 3*c*). No precipitin line was observed when normal plasma was used as antigen (Fig. 3*b*). The immunoelectrophoretic patterns shown in Fig. 3 were obtained with unabsorbed antiserum obtained from rabbits less than 20 days after the first immunization.

Several additional precipitin lines were seen when crude factor VIII concentrates were run against antiserum drawn from rabbits more than 20 days after the first antigenic stimulus. These include immunoglobulin lines which are removed by absorption of the antiserum with normal serum. The factor VIII line is not removed by a single absorption with normal or hemophilic plasma or serum, performed as described in Methods.

Analysis of purified factor VIII by acrylamide gel electrophoresis. The purified factor VIII preparation does not enter 5% polyacrylamide gels electrophoresed at pH 8.4 for periods up to 22 hr. Coomassie blue stain of these gels shows a band of protein concentrated between stacking and running gel. In order to confirm this point, a crude chymotryptic digest of Hyland method IV was electrophoresed on a standard 5% acrylamide gel for 7 hr and factor VIII assay was performed on 2-mm slices cut from the gel and crushed in buffer. Procoagulant activity was detected only in the top 2 mm nearest the stacking gel.

The purified factor VIII preparation was also electrophoresed on 5% acrylamide-0.1% SDS gels after

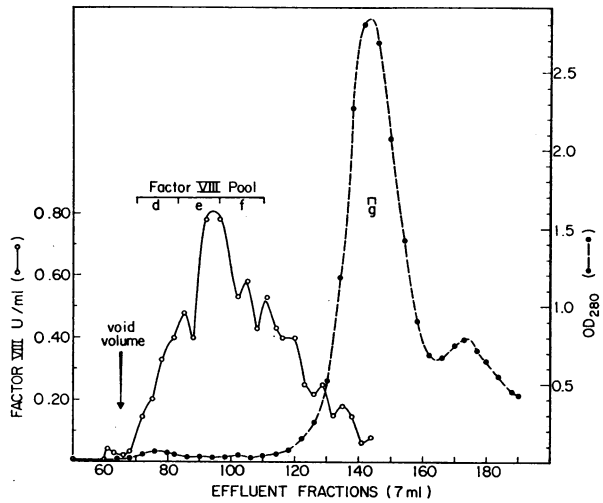

FIGURE 2 *Purification of factor VIII by gel filtration of α-chymotrypsin-digested Hyland method IV.* Hyland method IV (750 mg protein/25 ml, factor VIII 8 U/ml) was digested with α-chymotrypsin as described and applied to a Sepharose 4B column (5 × 100 cm). The column was eluted at 4°C with 0.05 M Tris-0.1 M NaCl, pH 7.4. Protein (●---●) was estimated by absorption at 280 mμ. Factor VIII (○——○) was assayed by the one-stage method.

TABLE I
*Summary of Steps in Purification of Factor VIII**

	Protein concn.‡	Vol.	Total protein‡	Factor VIII	Total VIII	Specific activity
		ml	*mg*	*U/ml*	*U*	*U VIII/mg protein*
1) Plasma	70 mg/ml	—	—	1	—	0.014
2) Hyland method IV AHG	30 mg/ml	25	750	8	200	0.27
3) Chymotrypsin digest of (2)	30 mg/ml	25	750	8	200	0.27
4) Sepharose eluate: *peak* factor VIII fraction	~8 μg/ml	—	—	0.8	—	100
5) Factor VIII pool	~8 μg/ml	280	2.24	0.48	135	60
6) Factor VIII pool conc. 250 times	2.0 mg/ml	1.1	2.20	12.8	14	6.4
7) Lyophilized powder	—	—	2.2–2.8	—	—	—

* See text for explanation of purification procedure.
‡ Measured by the Lowry method (19).

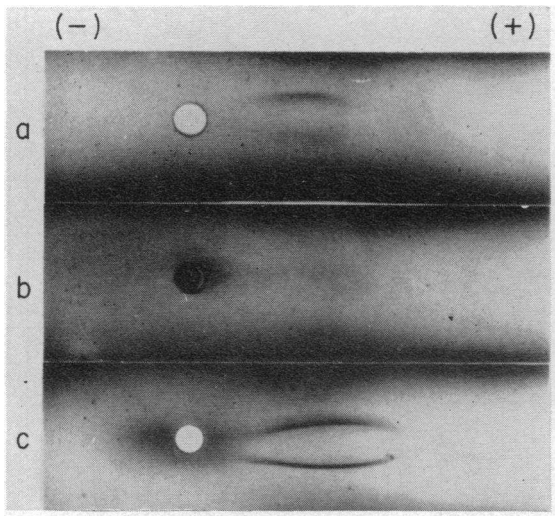

FIGURE 3 *Immunoelectrophoresis of crude and purified factor VIII.* Electrophoresis was performed in 0.05 M veronal buffer, pH 8.6, for 2 hr at 40 mv on 1.5% ionagar plates. Immunodiffusion was carried out for 40 hr at room temperature, using rabbit antiserum to purified factor VIII. Antigens: (*a*) factor VIII pool from Sepharose column (Fig. 2) concentrated 10 times; (*b*) normal plasma; (*c*) Hyland method IV after chymotrypsin digestion as described in Fig. 1*a*.

preincubation of the sample with 1% SDS in the presence or absence of the sulfhydryl-reducing agent dithioerythritol. The electrophoretic pattern was compared with that of similarly treated Hyland method IV before and after chymotryptic digestion and with that of purified fibrinogen. Molecular weights of the observed protein bands were estimated by comparison of their migration in SDS-acrylamide gels with the migration in the same system of proteins of known molecular weight (see Methods).

In the absence of reducing agent, the bulk of protein in the factor VIII preparation does not enter the SDS-acrylamide gel (Fig. 4*d*). This is in contrast to Hyland method IV (Fig. 4*b*) which forms a broad band at the position of fibrinogen (Fig. 4*a*), and to the chymotrypsin-digested Hyland method IV (Fig. 4*c*) in which the major band is a large digestion product of fibrinogen. Fig. 5 compares the migration pattern of the same samples after incubation in 1% SDS and 5 mM dithioerythritol. The factor VIII complex is dissociated in the presence of SDS and disulfide-reducing agent into subunits which can now enter the SDS-acrylamide gel (Fig. 5*d*). A major band of approximately 240,000 plus four minor bands with molecular weights between 80,000 and 160,000 are formed. Both major and minor bands stain with PAS. Under the same conditions, fibrinogen is dissociated into its three polypeptide chains (α, β, and γ) of approximately 79,000, 63,000, and 53,000 molecular weight, respectively (Fig. 5*a*). Hyland method IV shows similar bands, but there is additional high molecular weight material (Fig. 5*b*). The chymotrypsin-digested Hyland method IV (Fig. 5*c*) does not show this material; most of the protein in the digest migrates to the position of the polypeptide chains of fibrinogen, but the individual chains are no longer discernable.

Fig. 6A and B show SDS-acrylamide gels of successive fractions eluted from Sepharose. In the absence of reducing agent (Fig. 6A) almost all of the protein in the factor VIII pool is in a high molecular weight complex and does not enter the gel. In the presence of reducing agent (Fig. 6B), a major band of 240,000 mol wt appears first, followed by the minor bands apparently in relation to protein load.

Composition of purified factor VIII. Fibrinogen was not present in the purified factor VIII preparations as measured by immunodiffusion against rabbit antifibrinogen or by polyacrylamide gel electrophoresis. The sensitivity of these methods permits the conclusion that less than 5% of the protein present is fibrinogen.

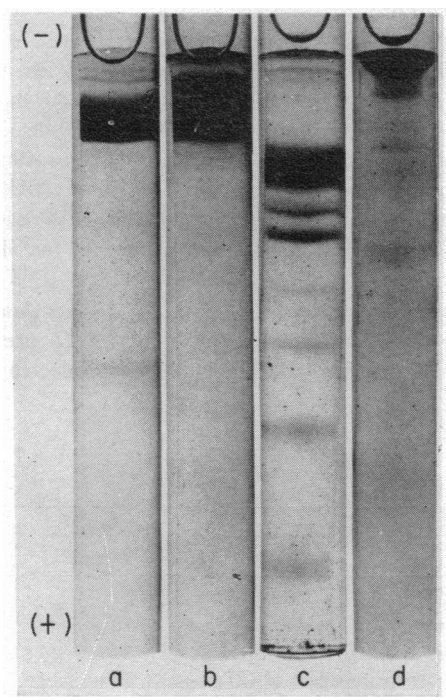

FIGURE 4 *SDS-acrylamide gel electrophoresis before disulfide reduction.* Samples were preincubated in 1% SDS-0.1 M sodium phosphate buffer (pH 7.1) with 0.025% sodium azide, for 3 hr at 37°C. Electrophoresis was carried out on 5% acrylamide-0.1% SDS gels in 0.1% SDS-0.1 M sodium phosphate, pH 7.1, for 3 hr at 9.6 ma/gel. *a*) fibrinogen; *b*) Hyland method IV AHG; *c*) '*b*' after chymotrypsin digestion; *d*) lyophilized factor VIII pool from Sepharose gel filtration (see Fig. 2 and Table I).

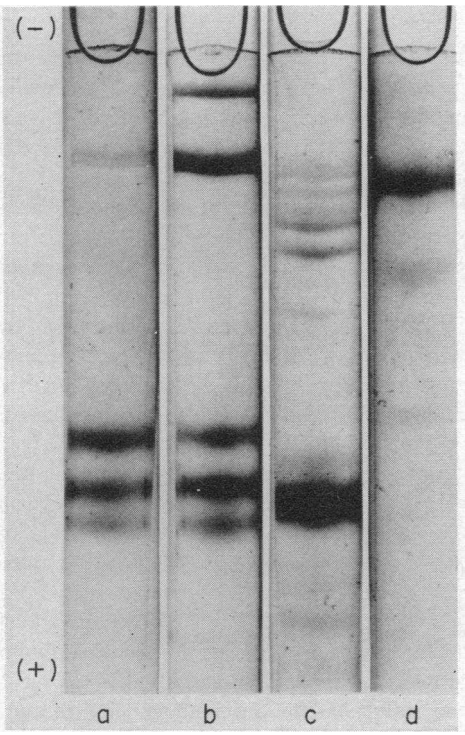

FIGURE 5 *SDS-acrylamide gel electrophoresis after disulfide reduction.* Conditions were as described in Fig. 4 except that samples were preincubated in the presence of 5 mM dithioerythritol. *a*) fibrinogen; *b*) Hyland method IV AHG; *c*)'*b*' after chymotrypsin digestion; *d*) lyophilized factor VIII pool from Sepharose gel filtration (see Fig. 2 and Table I).

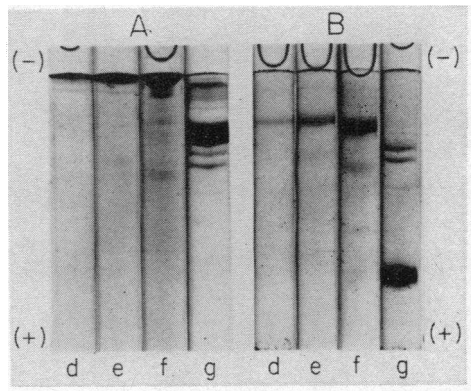

FIGURE 6 *SDS-acrylamide gel electrophoresis of eluate fractions from gel filtration.* Hyland method IV AHG was digested with chymotrypsin and filtered on Sepharose 4B as described in Figs. 1a and 2. Pooled fractions (Fig. 2: *d, e, f,* and *g*) were concentrated and lyophilized separately, incubated in 1.0% SDS-0.1 M sodium phosphate, pH 7.1, in the presence or absence of 5 mM dithioerythritol, and electrophoresed on 0.1% SDS-5% acrylamide gels for 3 hr. A) samples incubated in absence of reducing agent. B) samples incubated with 5 mM dithioerythritol. As shown in Fig. 2, fractions *d, e,* and *f* are from the factor VIII pool, and fraction *g* is from the protein peak.

13 mg of purified lyophilized factor VIII protein prepared from Hyland method IV were analyzed for sialic acid, hexose, hexosamine, amino acids, and lipid. The results were compared with similar analysis of 14 mg of the Hyland method IV starting material, and with lyophilized buffer eluted from the column.

The sialic acid content of the factor VIII preparation was found to be 0.98% compared with 0.7% for the Hyland method IV, which is similar to values reported for fibrinogen (22, 23). Glucosamine and galactosamine were determined on a 6 N HCl hydrolysate on the amino acid analyzer and were found to be 2.7% and 0.17% respectively. Hyland method IV contained 2.2% glucosamine and 0.4% galactosamine, Mosesson, Alkjaersig, Sweet, and Sherry (23) reports a slightly lower total hexosamine of 1.1% for fibrinogen. Qualitative hexose analysis of factor VIII by paper chromatography showed mannose, galactose, and fucose to be present in approximately 2:2:1 ratio; these were estimated to be 1–2% of the protein content. Only mannose and galactose could be detected in Hyland method IV; these were estimated to be 1% of the protein content, similar to the findings of others (23, 24) for fibrinogen.

Amino acid analysis was performed on three separate preparations of purified factor VIII. The results of these analyses are shown in Table II.

TABLE II
Amino Acid Analysis of Purified Factor VIII

Amino acid	*mmoles/mg protein*
Aspartic	1.03
Glutamic	0.946
Threonine	0.461
Serine	0.700
Proline	0.644
Alanine	0.506
Glycine	0.772
Valine	0.639
Methionine	0.087
Isoleucine	0.345
Leucine	0.488
Tyrosine	0.193
Phenylalanine	0.253
Lysine	0.437
Arginine	0.358

Lyophilized samples were suspended in 6 N HCl, sealed, evacuated, and hydrolyzed for 20 hr at 110°C. Figures given are averages of determinations on three separate samples. Half-cystine was present but not quantitated. Histidine and tryptophan were not measured.

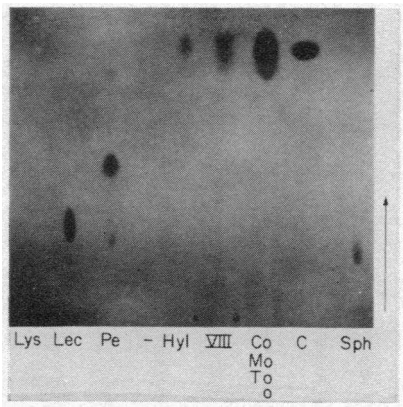

FIGURE 7 *Lipid analysis by thin-layer chromatography.* Samples were applied to silica gel plates and chromatographed for 45 min in chloroform-methanol-H_2O 65 : 25 : 4. 'Hyl' and 'VIII' are chloroform-methanol extracts of Hyland method IV and purified factor VIII, respectively. Standards: C, cholesterol; Co, cholesterol oleate; Mo, methyl oleate; To, triolein; o, oleic acid; Pe, phosphoethanolamine; Lec, lecithin; Lys, lysolecithin; Sph, sphingomyelin.

Analysis of lipids in the factor VIII preparation was performed by thin-layer chromatography of chloroform-methanol extracts, using a variety of solvents in order to identify both polar and nonpolar components. The factor VIII preparation contained small amounts of nonpolar lipid, provisionally identified as cholesterol and fatty acid, as well as a third unidentified component. These were estimated to comprise less than 5% of the protein content. Hyland method IV contained smaller amounts of the same lipid components. No polar lipids, and in particular none of the common phospholipids was found to be present in either the purified factor VIII or Hyland method IV. This is shown in Fig. 7.

Purification of factor VIII from cryoprecipitate. Cryoprecipitate from 2 U of plasma (total 75 U factor VIII/1200 mg protein per 24 ml) was digested with chymotrypsin until the fibrinogen was no longer coagulable, as shown in Fig. 1*b*. The factor VIII level remained at 3 U/ml. The digest was filtered through Sepharose 4B, and an elution pattern was obtained which was similar to that of the Hyland IV digest, except that the maximum factor VIII activity was 0.1 U/ml, and total protein yield of the purified factor VIII protein was approximately 0.75 mg.

SDS-acrylamide gel electrophoresis of this material after disulfide reduction in 1% SDS showed the same major band at approximately 240,000 mol wt as shown for the Hyland method IV-derived factor VIII in Fig. 4*d*. No further analysis has been performed on this preparation.

DISCUSSION

Our studies indicate that factor VIII is a macromolecular glycoprotein. The intact molecule in its purified form has a molecular weight of at least 2×10^6 based on gel filtration studies, and a principal subunit of 240,000 mol wt.

However, the significance of the large molecular size of factor VIII in its purified form is not clear. It is possible that factor VIII exists in plasma in a monomeric form complexed with other proteins, and that the large size of the purified molecule represents aggregation of the monomers when other proteins are removed. In this regard, the broad elution pattern of factor VIII seen in Fig. 2 may represent elution of aggregates of different sizes. This point will be discussed further below.

The suggestion has been made that factor VIII is a glycoprotein, based on its precipitation by concanavalin A, (8) and the staining of purified factor VIII fractions with PAS after electrophoresis (8, 14). The presence of substantial amounts of lipid in purified factor VIII was recently reported by Hershgold, Davison, and Janszen (6), and the suggestion was made that phospholipids are required for its procoagulant activity (25). Thus, despite some uncertainty as to the homogeneity of our factor VIII preparation, it seemed of interest to carry out analyses for amino acids, carbohydrate, and lipid.

The amino acid analysis shows no unusual features. There is good agreement between analyses of different preparations, and between the leading and trailing portions of the factor VIII peak from the Sepharose column. There are no major differences between the present amino acid analysis of factor VIII and that reported by Hershgold (6). The low absorption at 280 mμ noted by many investigators for their purified factor VIII preparations was probably a reflection of low concentration rather than of an unusually low content of aromatic amino acids.

Carbohydrate present in the factor VIII preparation includes 1% sialic acid, 2.8% hexosamine, and approximately 1–2% hexose, (mannose, galactose, and fucose). Thus, by our measurements, factor VIII falls into the category of most secreted proteins, which contain small amounts of carbohydrate (26). The presence of mannose explains the precipitation of factor VIII by concanavalin A, which binds molecules bearing mannose and glucose. McKee (13) and Hershgold et al. (6) reported hexose contents of 5% and 10% respectively for their purified factor VIII preparations.

Lipid analysis of our factor VIII preparation showed small amounts of nonpolar lipid (estimated to be less than 5% of the protein content) consisting of cholesterol, fatty acids, and possibly triglyceride. No phospholipids were present. These findings are in striking contrast to those of Hershgold et al. (6, 25) who report that their

factor VIII preparation contains 11% lipid, including large amounts of phospholipid, and that phospholipases appear to play a role in the activation and inactivation of factor VIII.

In this regard, Ratnoff, Kass, and Lang (10) found that the lipid content of their Sepharose-purified factor VIII was dependent on the chylomicron content of the starting plasma and concluded that the small amounts of lipid in the final preparations were contaminants. Green (12) found no lipid in his factor VIII preparations purified by gel filtration of cryoprecipitate.

The subunit structure of purified factor VIII has been studied by acrylamide gel electrophoresis in SDS. The material remains in aggregated form when denatured in SDS alone, and does not enter the gel unless preincubation in SDS includes a sulfhydryl reducing agent. After sulfhydryl reduction, dissociation of the complex occurs, resulting in a major subunit of 240,000 mol wt. This would be considered large for a single polypeptide chain, since most well characterized proteins have polypeptide subunits of less than 100,000 mol wt. However, it appears that as more complex proteins are analyzed, larger subunits are described. Trayer, Yasuhiko, Reynolds, and Tanford (27) have recently described membrane proteins with polypeptide subunit sizes up to 220,000.

In addition to the major subunit of 240,000 mol wt, there are three to four minor bands seen on SDS gels. These may represent subunits of contaminating proteins; however, since almost none of the SDS-treated protein enters the gel in the absence of sulfhydryl reducing agent, these contaminants would have to be macromolecules with disulfide-bonded subunits.

Another explanation for the multiple banding pattern of purified factor VIII is that the native molecule is composed of more than one type of polypeptide chain. It is also possible that chymotrypsin, while not altering the biologic activity of factor VIII, may have produced "nicks" in a single polypeptide chain. These breaks might not be evident in the native molecule, due to noncovalent interactions between polypeptide segments which hold the molecule together. However, upon reduction and denaturation of the molecule in SDS, the polypeptide fragments would dissociate and produce multiple banding patterns on acrylamide gels.

McKee (13) has stated that his purified factor VIII, prepared without the use of proteolytic enzymes, has a banding pattern on SDS gels similar to that reported here. In contrast, Hershgold (28) has reported that his purified factor VIII has a 22,000 mol wt subunit size on SDS gels, and a mol wt of 30,000 by fingerprinting of tryptic peptides.

Several artifacts of the SDS gel system must be considered when attempting to identify subunits of a protein by this method. The first is that incomplete dissociation of the protein into subunits may occur, producing a multiple banding pattern which is artifactual; the second is that bacterial contamination and/or enzymatic activity may produce spurious dissociation of the protein into small subunits (29).

Antibody developed in rabbits to the purified factor VIII preparation produces a single precipitin band on immunoelectrophoresis when reacted with the purified antigen, suggesting a high degree of homogeneity of the factor VIII antigen. The presence of additional minor precipitin lines when antibody is reacted with crude antigen indicates that the purified factor VIII preparation contains small amounts of contaminating proteins which induce antibody formation but which are present in quantities too small to be seen by direct precipitation of antibody and purified antigen. Rabbit antibodies to factor VIII made by Zimmerman, Ratnoff, and Powell (30) and Hershgold et al. (6) gave similar immunoelectrophoretic patterns. As in our study, their antibodies gave no precipitin line using plasma as antigen. The explanation for this may be simply that the levels of factor VIII in plasma are too low to form visible antigen-antibody precipitates in agar.

Antibody made in goats to crude factor VIII (Hyland method IV AHG) in our laboratory (31) produced an immunoelectrophoretic pattern with factor VIII similar to the rabbit antibody. It is interesting to note, however, that the rabbit antibody, which was directed toward purified, inactive factor VIII, had poor procoagulant-neutralizing activity, while the goat antibody, which was developed to crude, highly active factor VIII had excellent neutralizing activity.

Important biological questions remain concerning the form which factor VIII takes in plasma, the nature of its activation and of the procoagulant activity itself. It is possible that factor VIII has a specific molecular interaction with fibrinogen in plasma, as has been suggested by others (32–34). This would explain the difficulty encountered in separating fibrinogen from factor VIII during purification. The removal of fibrinogen during the purification procedure might then cause aggregation of monomeric factor VIII units, resulting in the large macromolecular complexes characteristic of purified factor VIII. Since removal of protein during purification markedly decreases the stability of factor VIII activity, it might be postulated that interaction with protein (fibrinogen) is important in maintaining a specific conformation which is susceptible to activation during clotting.

Thrombin in trace amounts has been shown to activate factor VIII (35, 36). It was suggested by Biggs, MacFarlane, Denson, and Ash (32) that thrombin activates

factor VIII in part by release of factor VIII from fibrinogen during cleavage of the fibrinopeptides. The action of chymotrypsin in the present experiments might be seen as an analagous to that of thrombin. However, in contrast to thrombin, chymotrypsin renders fibrinogen incoagulable, and activation of factor VIII (occasionally observed) is minor in degree. This analogy may help to explain the usefulness of α-chymotrypsin in the preparation of fibrinogen-free factor VIII.

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