The Subunit Structure of Normal and Hemophilic Factor VIII

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A B S T R A C T Human factor VIII from normals and
hemophiliacs was partially purified by ethanol and poly-
ethylene glycol precipitations. Final purification was
achieved by gel filtration on 2 or 4% agarose or ion
exchange chromatography on diethylaminoethyl cellu-
lose. Comparable amounts of highly purified protein
were obtained from normal and hemophilic plasma fol-
lowing the agarose chromatography step. Highly puri-
fied factor VIII was not dissociated by 6 M guanidine
hydrochloride or 1% sodium dodecyl sulfate. However,
when reduced by β-mercaptoethanol and analyzed by
sodium dodecyl sulfate polyacrylamide gel electrophore-
sis, a single subunit species with an estimated 195,000
molecular weight was found for both normal and hemo-
philic factor VIII. By sedimentation equilibrium analy-
sis, the normal factor VIII subunit was homogeneous
and had an estimated molecular weight of 202,000. The
subunit polypeptides from normal or hemophilic factor
VIII contained carbohydrate. Each was homogeneous
by isoelectric focusing. Immunodiffusion of purified
normal and hemophilic factor VIII against rabbit anti-
serum to purified normal human factor VIII showed
a single line of precipitation. Very low concentrations
of purified human thrombin initially increased the
activity of normal factor VIII about threefold and then
progressively destroyed activity by 3 h. Only minimal
activation occurred with hemophilic factor VIII. Both
the activation and inactivation of normal and hemo-
philic factor VIII were unaccompanied by detectable
changes in subunit molecular weight. These findings
may have implications for the definition of the molecu-
lar defect in hemophilic factor VIII.

INTRODUCTION

Several investigators have shown that highly purified
human factor VIII (antihemophilic factor; AHF [1])
can be eluted near the void volume when factor VIII
concentrates are filtered through 2 or 4% agarose col-
umns (1–11). These studies suggested that factor VIII
has a molecular weight of approximately 2 million, but
aggregation as a possible explanation for this high
molecular weight cannot be entirely excluded. It has
been shown, however, that even in dissociating solvents
factor VIII has a molecular weight at least in excess
of 400,000 (5) and an estimated $s_{25,w}$ value of 16.3 (8).
Human factor VIII is known to be a glycoprotein,
containing about 5–10% carbohydrate (8). Recently it
has been suggested that bovine factor VIII is likewise
a glycoprotein with a molecular weight in excess of one
million and is composed of disulfide linked subunits, the
smallest of which may be about 85,000 in molecular
weight (10). While some of the physicochemical prop-
erties of human factor VIII have been determined
(2–4, 8–11), the study of its subunit structure has only
been recently approached (5, 10–12). Particularly in
light of the immunological evidence that hemophilic
plasma contains nonfunctional factor VIII in quantities
comparable to those of functional factor VIII in normal
plasma (13–18), the characterization of the structures

[1] *Abbreviations used in this paper:* AHF, antihemophilic
factor; MW, molecular weight; $s_{25,w}$, sedimentation co-
efficient; SDS, sodium dodecyl sulfate; $\bar{v}$, partial specific
volume.

of normal and nonfunctional factor VIII could be useful in defining the molecular defect in hemophilia.

We now wish to report the results of studies which show that normal human factor VIII is composed of an undetermined number of large subunits which are homogeneous by size and electrical charge. Normal factor VIII was also compared with hemophilic factor VIII with respect to subunit molecular weight, carbohydrate staining properties, and isoelectric focusing mobility. In addition, the effect of thrombin on the structure of both normal and hemophilic factor VIII has been examined.

METHODS

Factor VIII assay. Factor VIII activity was measured by two methods: (a) the one-stage partial thromboplastin time according to the technique of Langdell, Wagner, and Brinkhous (19), except that the hemophilic substrate plasma was "activated" by adding 5 mg kaolin (J. T. Baker Chemical Co., Phillipsburg, N. J.) to each ml of hemophilic substrate plasma and then incubating for 5 min with stirring at 37°C; Thrombofax (Ortho Laboratories, Raritan, N. J.) served as the source of cephalin in this assay; and (b) a modified thromboplastin generation test as described by Newman, Johnson, Karpatkin, and Puszkin (20). Factor VIII elution patterns during gel chromatography were monitored by determining clotting times for undiluted samples in the one-stage kaolin-activated partial thromboplastin time. Although the eluting buffer had an ionic strength of 0.3, the final ionic strength of the clotting assay mixture was 0.155; final ionic strengths ranging from 0.13 to 0.17 did not affect the clotting times of the assay mixtures. The reference plasma used in these assays was lyophilized pooled plasma standardized with respect to factor VIII content (courtesy of Dr. D. L. Aronson, Division of Biologics Standards, National Institutes of Health) and a fresh-frozen house standard plasma, repeatedly assayed against the reference plasma. Units of factor VIII activity were calculated by defining 1 U of factor VIII as that amount contained in 1 ml of normal male plasma (21).

Protein concentrations. These were estimated by the absorbance at 280 nm or by the method of Waddell (22). Where more quantitative measurements were indicated, the method of Lowry, Rosebrough, Farr, and Randall (23) was used.

Partial purification of normal factor VIII. The starting material in these experiments was either fresh frozen plasma, cryoprecipitate or glycine-precipitated factor VIII (Method IV, Hyland Div., Travenol Laboratories, Costa Mesa, Calif.). In each case, partially purified factor VIII was prepared by the method of Newman et al. (20) modifications being: that glycine-precipitated factor VIII concentrates were not subjected to the 3% ethanol precipitation step, but were introduced into the purification scheme at the 0.05 M Tris hydrochloride extraction step; and that a bentonite adsorption step was utilized to decrease fibrinogen content prior to precipitation with polyethylene glycol. This latter step 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 of Newman et al. (20). This mixture was incubated at room temperature and stirred slowly for 5 min and then centrifuged in polycarbonate bottles at 6,000 g for 15 min at 20°C. The

remaining precipitation steps as described by Newman et al. (20) were then carried out on the supernatant fluid. The final precipitate was dissolved in 0.30 M sodium chloride-0.05 M Tris hydrochloride, pH 7.4 to a final protein concentration of 30 mg/ml. This partially purified factor VIII will henceforth be called *Step 1* factor VIII.

Gel filtration. Gel chromatography was performed at 4°C on both 2 and 4% agarose columns (Sepharose 2B and 4B, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). A siliconized glass column, 5 × 92 cm, was used in these experiments. The void volume was estimated by filtration of an appropriate volume of 0.005% Blue Dextran 2000 (Pharmacia Fine Chemicals) in 4% sucrose. The volume of the partially purified factor VIII applied to the column was always 2–3% of the column volume. Elution was performed with 0.05 M Tris hydrochloride-0.3 M sodium chloride buffer, pH 7.4, at a pressure of approximately 40 cm. The flow rate averaged 18 ml/h and 4.2-ml fractions were collected in siliconized glass tubes. The factor VIII eluted from the agarose column will be referred to as *Step 2* factor VIII. Each eluant fraction was assayed immediately for factor VIII activity and its absorbance at 280 nm determined. As noted by Ratnoff, Kass, and Lang, the recovery of factor VIII from the agarose column improved with use (4).

DEAE chromatography. Step 1 normal factor VIII was dissolved in and dialyzed against the starting buffer, 0.05 M Tris, pH 7.35. Approximately 24 mg of Step 1 normal factor VIII were applied to a 0.9 × 10 cm DEAE-cellulose column equilibrated with the starting buffer. The column was developed with a 150 ml linear gradient, the limit buffer being 0.05 M Tris-0.50 M sodium chloride, pH 7.35. A peak of factor VIII activity was eluted reproducibly at 0.15–0.17 M sodium chloride; this material will be referred to as Step 2-DEAE factor VIII. Selected fractions containing factor VIII activity were pooled, dialyzed against water to remove salt and lyophilized prior to electrophoretic analysis.

Electrophoresis. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was carried out essentially as described earlier (24–26). Approximately 100 µg of lyophilized Step 2 factor VIII from each gel chromatography experiment or Step 2-DEAE normal factor VIII was dissolved in 0.10 ml of 1% SDS-5 M urea in 0.01 sodium phosphate buffer, pH 7.1, and one-half of this solution was reduced with a final concentration of 0.1 M β-mercaptoethanol. Each of the non-reduced and reduced samples were then incubated in the 1% SDS-5 M urea and 1% SDS-5 M urea-0.1 M β-mercaptoethanol solvents, respectively, at 37°C for 12–24 h following which the entire volume of each was applied to separate, single electrophoretic gels. In most of these studies, 5% polyacrylamide gels in the presence of 0.1% SDS were prepared in 15 × 0.6 cm glass columns. Alternatively, glass columns 10 cm in length were used. The electrophoretic buffer consisted of 0.1% SDS in 0.1 M sodium phosphate buffer, pH 7.1. All electrophoreses were performed at room temperature using a constant current of 6 mA per gel for about 6 h at which time the bromophenol blue dye marker (0.05%) had migrated within 1–2 cm of the gel bottom. Following exposure to thrombin, the factor VIII subunit was examined for changes in molecular weight by the high resolution 3.36% polyacrylamide gel-SDS method (27) as well as on 5% polyacrylamide gels in SDS. Gels were stained for protein with freshly prepared Coomassie brilliant blue (Colab Laboratories, Inc., Glenwood, Ill.) and were de-

stained as previously described (25). The subunit molecular weights of factor VIII were calculated from their electrophoretic mobilities and the experimentally determined mobilities of proteins of known molecular weight on 5% SDS-gels. The following proteins were reduced by β-mercaptoethanol and used as standards (the assumed molecular weights are given in parentheses; references are given for those molecular weights about which some controversy exists): rabbit muscle myosin (194,000) (28), β-galactosidase (120,000) (29), phosphorylase A (92,000), human transferrin (77,000) (30), bovine serum albumin (69,000), catalase (60,000), ovalbumin (43,000), pepsin (35,000), and chymotrypsinogen (26,000). Following electrophoresis, certain of the gels containing highly purified normal or hemophilic factor VIII were stained for carbohydrate-containing proteins with the periodic acid-Schiff reagent (31). This method was modified slightly in that after staining, the gels were washed overnight in distilled water and then destained in 50% methanol for 8 h. The gels were allowed to re-swell in 7% acetic acid and stored in that solution.

Amino acid composition. Duplicate samples of Step 2 normal factor VIII were hydrolyzed in 6 M HCl at 110°C, *in vacuo*, for 24, 48, 72, and 96 h. The hydrolysates were dried by rotatory evaporation *in vacuo*, dissolved in distilled water to give a final concentration of 0.25 mg/ml, and then analyzed quantitatively using a Beckman 120B amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with high sensitivity cuvettes. Half-cystine and methionine contents were determined after oxidation by performic acid to cysteic acid and methionine sulphone, respectively (32). Valine and isoleucine were quantitated from the analysis of 120-h hydrolysates. Tryptophan was not determined.

Sedimentation equilibrium analysis. A Beckman-Spinco Model E analytical ultracentrifuge was used in these studies. For the determination of subunit molecular weight, the high speed method of Yphantis (33) was used. Purified normal factor VIII was dissolved in 5.6 M guanidine hydrochloride-0.1 M β-mercaptoethanol to give a protein concentration of approximately 0.30 mg/ml. The speed employed was 20,000 rpm, the column height was 2.5 mm, and the temperature was 25±1°C. The subunit molecular weight was estimated by using a partial specific volume ($\bar{v}$) of 0.722 which was calculated from the amino acid composition (34) as well as at a $\bar{v}$ value of 0.712 which is the calculated $\bar{v}$ after correction for preferential binding of guanidine hydrochloride (35). Because of the difficulty in judging the exact value for $\bar{v}$, the subunit molecular weight of human factor VIII was also calculated at $\bar{v}$ values of 0.71 and 0.73. Other operations in these analyses have been described (36, 37).

Purification of factor VIII from normal and hemophilic plasmas. Following preliminary characterization of normal factor VIII, a series of experiments were performed to compare individual samples of factor VIII prepared from several normal plasmas and factor VIII-like material prepared from several hemophilic plasmas. Venous blood was obtained from each of six normal male volunteers and six hemophiliacs with factor VIII levels 80–120% and < 1–5% of normal, respectively, as measured by the method of Langdell et al. (19). Each 360 ml blood sample was collected into 40 ml of cold 3.5% sodium citrate and immediately centrifuged at 5,000 *g* at 4°C for 15 min. The supernatant plasma was siphoned from each specimen into separate plastic containers and frozen in a dry ice-ethanol bath. The samples were stored at −20°C and the modified

method for the partial purification of factor VIII was carried out on each sample at a convenient time, ranging from 1 to 21 days after collection. Each of the final precipitates resulting from the partial purification of factor VIII was dissolved in 0.30 M sodium chloride-0.05 M Tris hydrochloride buffer, pH 7.4 to give an average protein concentration of 27.5±2.8 mg/ml. The individual samples of Step 1 factor VIII from each normal or hemophilic plasma were then applied to a 4% agarose column (0.9 × 56 cm, siliconized) and eluted at 4°C with a pressure head of approximately 40 cm, using the 0.30 M sodium chloride-0.05 M Tris hydrochloride, pH 7.4, buffer. In each case, the sample volume applied to the column was 0.9 ml, and 0.9 ml fractions were collected into siliconized glass tubes. Fractions were assayed for factor VIII activity using the one-stage method and protein concentration estimated from the absorbance at 280 nm.

In the case of each normal sample, the eluant fractions which contained the void volume protein peak with factor VIII activity were pooled and dialyzed at 4°C against four changes of distilled water in a ratio of 1 part sample to 2,000 parts water. The samples were then quick-frozen and lyophilized in siliconized, conical 15 ml centrifuge tubes. The protein peak in each of the hemophilic elution patterns which corresponded to the active peak in the normal elution patterns was isolated likewise. Each sample was then prepared for analysis by SDS-gel electrophoresis with and without β-mercaptoethanol as outlined under *Electrophoresis.*

Isoelectric focusing. Highly purified normal and hemophilic factor VIII preparations were reduced and carboxymethylated according to the method of Hirs (38) except that 6 M guanidine hydrochloride in 0.55 M Tris HCl buffer, pH 8.1, containing 0.5 M EDTA was used as the solvent. Following dialysis against distilled water, the samples were concentrated by lyophilization and then analyzed by isoelectric focusing on polyacrylamide gels by the method of Hayes and Wellner (39), modified by reducing the riboflavin concentration from 0.0015 to 0.0005% since polymerization did not occur at the higher concentration.

Immunology. To insure that the activity of purified fractions was due to the specific presence of factor VIII, activity was determined before and after incubation with plasma from a hemophiliac with a known specific factor VIII inhibitor titer of 1:512 as determined by using the partial thromboplastin time test (40). To demonstrate specific inhibition, 0.2 ml of inhibitor plasma was mixed with 0.2 ml of the most active void volume fractions eluted from the gel columns. Following incubation for 30 min at 37°C, 0.1 ml of this mixture was assayed by the modified one-stage partial thromboplastin time, using hemophilic plasma as substrate. In addition antiserum to normal human factor VIII was prepared by injecting an emulsion containing the most concentrated portion of the 4% agarose void volume protein (usually 200–300 μg in 2–3 ml Tris saline buffer) mixed with an equal volume of alumina C γ-Gel (A grade, Calbiochem, Los Angeles, Calif.) into the foot pads of rabbits. This was repeated in 3 wk, and 7 days after the final injection 20–30 cc of blood was drawn from an ear artery or by intracardiac puncture. The blood was allowed to clot in glass tubes at 37°C for at least 2 h, then centrifuged at 1,500 *g* for 20 min. Antiserum was stored in 1 ml aliquots at −15°C. Absorbed antiserum was prepared as described by Zimmerman, Ratnoff, and Powell (16) by using the supernatant fluid from the 3% ethanol cyoprecipitate as the absorbant. Step 2 normal and hemophilic factor VIII was prepared for immunodiffusion by concentrating
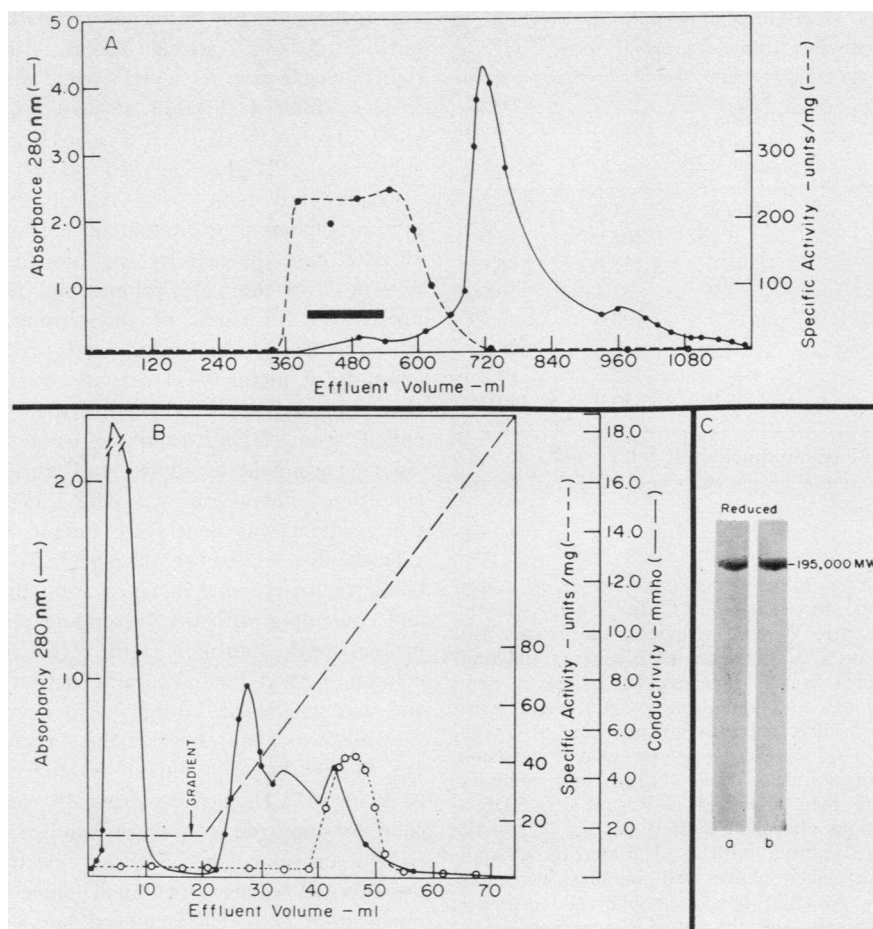
FIGURE 1 *Panel A.* 4% agarose gel filtration of partially purified factor VIII (Step 1 factor VIII) (column size: 5 × 92 cm); the fractions beneath the horizontal bar were pooled and contained purified factor VIII (Step 2 factor VIII). *Panel B.* Gradient elution of partially purified factor VIII (Step 1) from DEAE-cellulose (column size 0.9 × 10 cm). The material coinciding with the descending limb of the last protein peak, and which maximally shortened the activated partial thromboplastin time, contained purified factor VIII (Step 2- DEAE factor VIII). *Panel C.* SDS-gel electrophoresis of reduced factor VIII prepared from 4% agarose chromatography (gel *a*) or from DEAE-chromatography (gel *b*). MW, molecular weight.

the 4% agarose void volume protein in a collodion bag concentrator. By this method the initial 3–4 ml of the void volume could be reduced to a volume of 100–500 μl, but with an estimated 50% loss of protein. Immunodiffusion studies of Step 1 and Step 2 normal and hemophilic factor VIII were performed in agarose, B grade, prepared as previously described (16). In each case, the wells were filled three times with 10 μl of a solution of Step 1 or Step 2 normal or hemophilic factor VIII containing about 1 μg/ml.

Effect of thrombin on factor VIII. Using glycine-precipitated material, Step 1 normal factor VIII was prepared and further purified by elution from the 5 × 92 cm 4% agarose column. Approximately 100 mg of Step 1 material was dissolved in 12 ml and applied to the column. The Step 2 factor VIII that was eluted in the void volume peak was pooled and then divided into 4 ml aliquots in siliconized

tubes. The final protein concentration of the pooled factor VIII was 5.7 μg/ml; factor VIII activity was 0.52 U/ml. The 4-ml aliquots were arranged in eight pairs. Purified human thrombin (also supplied by Dr. D. L. Aronson) was added to one tube of each pair to give a final concentration of 0.05 NIH U/ml, and an equal volume of buffer was added to the other tube of each pair. The paired mixtures were incubated at 37°C and at the end of each of the following time periods: 0.25, 5, 10, 15, 30, 105, 195, and 284 min, a pair of aliquots was assayed for factor VIII activity using three dilutions in the kaolin-activated, one-stage partial thromboplastin method. In this assay, control clotting times were as follows: 168 s for buffer alone, 156 s for a buffer solution containing 0.05 NIH U/ml of thrombin, and 129 s for a 1:20 dilution of the purified normal factor VIII solution. Immediately after a sample for assay had been removed from an incubation

	Glycine-precipitated factor VIII concentrate*	Step 1	Step 2
Volume, ml	1,096.0	14.0	92.0
Activity, U/ml	3.93	302.0	13.0
Total units	4,308.0	4,228.0	1,196.0
Protein, mg/ml	4.9	33.4	0.063
Total protein, mg	5,414.0	468.0	5.79
Specific activity, U/mg	0.80	9.0	206.0
Yield, %	—	98.5	29.2
Purification-fold‡	55.9	630.0	14,400.0

* Dissolved in 0.02 M tris hydrochloride buffer, pH 7.4.

‡ With respect to a reference plasma containing 1 U factor VIII/ml and 70 mg protein/ml.

mixture, the remaining volume was quick-frozen in a dry ice-ethanol bath and stored at −80°C for electrophoretic analysis at a later date. When convenient, the frozen aliquots were placed in a 37°C water bath and as thawing began, 0.5 ml of 50% formic acid was mixed with each sample to lower the pH to 2 and thereby inactivate thrombin.[2] Each sample was dialyzed in a volume ratio of 1:200 against three changes of distilled water, previously adjusted to pH 2.0 with formic acid. After dialysis, the samples were lyophilized and then dissolved in 0.5 ml 1% SDS-5 M urea-0.01 M sodium phosphate buffer, pH 7.1. One-half of this solution was reduced with 2 μl β-mercaptoethanol and incubated as described above. All samples were examined in duplicate by SDS-gel electrophoresis, one gel being stained for protein and the other for carbohydrate. Aliquots of Step 2 hemophilic factor VIII were examined similarly by incubating paired solutions containing 6.6 μg/ml and <1% activity with either a final thrombin concentration of 0.05 NIH U/ml or with buffer only. At the completion of the incubation periods, each thrombin-exposed sample and its control were assayed and then prepared for SDS-gel electrophoresis as described above.

The effect of thrombin concentration on factor VIII activity, after a constant incubation time, was also studied. In this experiment 4-ml aliquots of purified factor VIII containing 1.5 U/ml were incubated for 1 min at 37°C with the following final concentrations of purified human thrombin: 0.03, 0.06, 0.12, 0.31, and 0.62 U/ml. At the end of the 1 min incubation period, each mixture was assayed by the two-stage method. It should be emphasized that the highest final concentration of thrombin used in the incubation mixtures did not affect the assay result when compared with a buffer control.

RESULTS

As previously reported (1–11), the partial purification of factor VIII by the method of Johnson, Newman, Howell, and Puszkin (2) followed by filtration through

4% agarose results in an approximate 7,000–10,000-fold purification over normal plasma. Figure 1A shows a typical chromatogram when Step 1 normal human factor VIII is filtered through 4% agarose. In this experiment factor VIII activity was calculated using the two-stage assay. Highly purified normal factor VIII was also prepared using fresh frozen plasma, cryoprecipitate, or glycine-precipitated concentrates of factor VIII. In each case the activity was associated with the protein peak in the void volume and the yield of active protein in this area of the chromatogram increased when material which contained progressively greater amounts of factor VIII activity was used. Besides gel chromatography, partially purified factor VIII was also eluted from DEAE-cellulose using a linear sodium chloride gradient as shown in Figure 1B. Under these conditions, fibrinogen was eluted in the first peak as the gradient was developed. Factor VIII activity was reproducibly eluted at 0.15–0.17 M sodium chloride, being removed some distance from the fibrinogen peak and coinciding with the descending portion of the third protein peak. Normal factor VIII prepared by this column method had a specific activity of 30–50 U/ml and was purified 2,100–3,500-fold over starting plasma; the yield was about 3.6% of the starting activity. Figure 1C shows that the eluant fractions which contained peak factor VIII activity from 4% agarose or DEAE-cellulose appeared as a single band of 195,000 molecular weight examined by SDS-gel electrophoresis in the presence of β-mercaptoethanol. Table I summarizes the purification and yield achieved by the 4% agarose gel filtration method.

Fig. 2 shows the mean elution patterns when partially purified factor VIII from the six normal and six hemophilic volunteers was eluted from 4% agarose. The number of absorbancy units of Step 1 hemophilic factor VIII applied to the column was always slightly less than the amount of Step 1 normal factor VIII. Despite this, when partially purified hemophilic factor VIII was applied to the column, the amount of protein eluted in the void volume was not significantly different from that observed when partially purified normal factor VIII was chromatographed. All of the hemophiliacs had very similar quantities of void volume protein. However, striking differences were observed in the factor VIII activity in this region of the chromatograms. In these experiments the percent shortening of the partial thromboplastin time of hemophilic plasma given by an eluate fraction when compared with a buffer control was divided by the absorbance at 280 nm to generate an arbitrarily defined specific activity. As shown in Fig. 2, the amount of factor VIII activity associated with the void volume region in the elution pattern of the normal factor VIII was very significantly
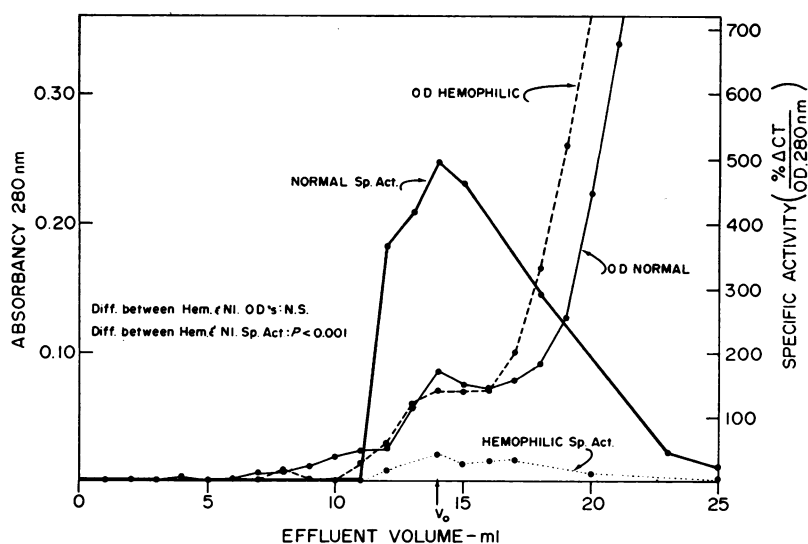
FIGURE 2 Comparison of the mean elution patterns and the mean specific activities of individual samples of Step 2 factor VIII prepared separately from six normal plasmas and six hemophilic plasmas by filtration through 4% agarose (column size 0.9 × 56 cm). The mean amount of normal protein which appeared in the void volume was essentially identical to the mean amount of hemophilic protein. The specific activities were significantly different. It should be noted that each of the hemophilic plasmas contained virtually the same amount of void volume protein.

greater ($P < 0.001$) than in the case of the hemophilic factor VIII. The differences in the mean amount of protein or the mean amount of factor VIII activity between the six individual normal when compared with the six individual hemophilic preparations were examined using a tube-to-tube comparison of eluate fractions as well as a comparison of areas beneath the protein or activity peaks. In each case the same statistical results were obtained.

Subunit molecular weight of factor VIII. Fig. 3 shows typical sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns of the protein peak associated with functional as well as nonfunctional factor VIII from gel filtration of normal and hemophilic concentrates. Gels *a* and *b,* respectively, show that nonreduced normal or hemophilic factor VIII does not penetrate the top of 5% gels despite denaturation by 2% SDS-5 M urea. Even after it was dissolved in 6 M guanidine hydrochloride and dialyzed into 2% SDS, neither protein entered 5% polyacrylamide gels. However, gels *c* and *d* show that after reduction of either normal or hemophilic factor VIII by 0.1 M β-mercaptoethanol, a major protein band of 195,000 molecular weight was observed in each case. Gels *e* and *f* show that this band, whether from normal or hemophilic protein, contained carbohydrate when stained with the periodic acid-Schiff reagent. It should be noted that despite reduction by concentrations of β-mercaptoethanol of 0.4 M for as long as 40 h in 2% SDS or 6 M

guanidine hydrochloride, no further decrease occurred in the molecular weight of this subunit species.

Figure 4*A* shows the standard curve for molecular weight determination by the SDS-gel electrophoretic method. By this technique both reduced normal and hemophilic factor VIII had molecular weights of 195,000. Figure 4*B* depicts the logarithmic relationship be-
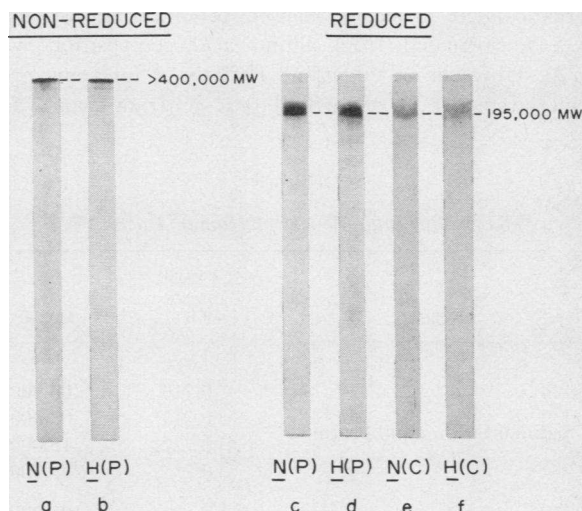

FIGURE 3 SDS-gel electrophoresis of non-reduced and reduced normal, *N*, and hemophilic, *H*, factor VIII. Gels indicated by (P) were stained for protein; gels indicated by (C) were stained for carbohydrate. MW, molecular weight.
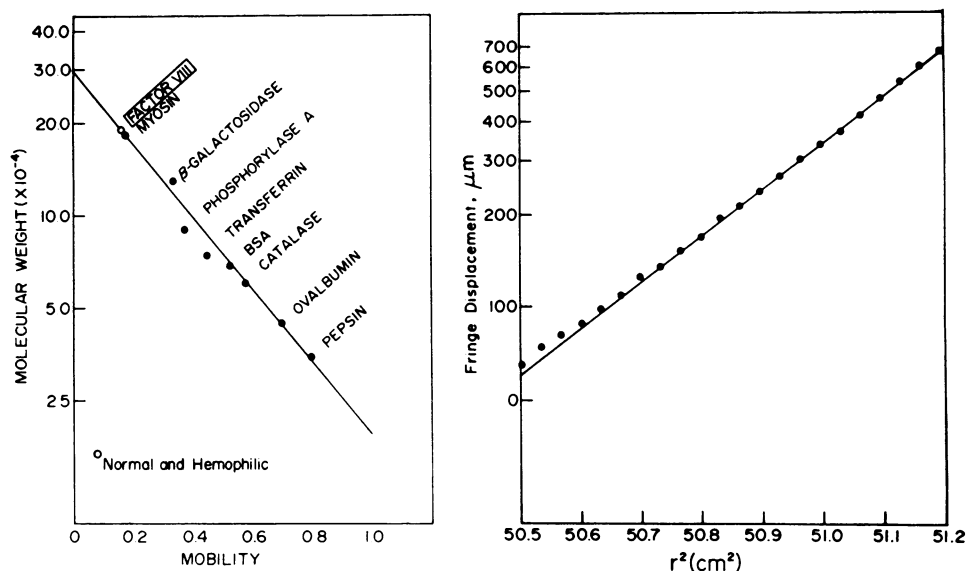
FIGURE 4 *Panel A.* SDS-gel electrophoretic standard curve for determination of the subunit molecular weight of reduced normal and hemophilic factor VIII. The mobilities of the normal and hemophilic factor VIII subunits were identical. *Panel B.* Sedimentation equilibrium of reduced normal factor VIII in 5.6 M guanidine hydrochloride. See text for details.

tween the fringe displacement and the square of the distance from the center of rotation when the factor VIII subunit is analyzed by sedimentation equilibrium ultracentrifugation. Although the linear relationship from cell bottom to less than 100 μm fringe displacement found by this technique indicates homogeneity of the factor VIII subunit, calculation of an exact molecular weight depends upon knowledge of the partial specific volume ($\bar{v}$) for the subunit. Therefore Table II gives a list of molecular weights for different $\bar{v}$ values. The $\bar{v}$ calculated from amino acid composition was 0.722. However in guanidine HCl the $\bar{v}$ for some proteins is smaller by about 0.01–0.02 cc/gram than $\bar{v}$ for

TABLE II

Subunit Molecular Weight of Human Factor VIII

Method	Partial specific volume, $\bar{v}$	Mol wt
	cc/g	
Sedimentation equilibrium	0.710	200,000
	0.712*	202,000
	0.722‡	215,000
	0.730	226,000
SDS-gel electrophoresis	—	195,000

* Corrected $\bar{v}$ for preferential binding of guanidine hydrochloride (34).
‡ Calculated from the amino acid composition (33).

the same protein in dilute aqueous salt solution (35). When arbitrarily corrected for this effect, the calculated molecular weight from sedimentation equilibrium data becomes $\sim$ 202,000 for the normal factor VIII subunit. This value is in good agreement with the 195,000 molecular weight obtained for both the normal and hemophilic factor VIII subunit by SDS-gel electrophoresis.

Isoelectric focusing. Fig. 5 shows superimposed scans of reduced and alkylated normal and hemophilic factor VIII when analyzed by isoelectric focusing in polyacrylamide gels. Although slightly diffuse, both the normal and hemophilic factor VIII subunits had identical mobilities when examined by this method.

Amino acid composition. Table III shows the amino acid analysis of normal human factor VIII. The values listed in the first column are the averages of each residue when calculated from the values obtained by analyses of duplicate samples at three different times of hydrolysis. Results are expressed in terms of residues of each amino acid per subunit molecular weight. The second column shows the amino acid composition of normal factor VIII reported by Hershgold, Davison, and Janszen (8), but recalculated on the basis of 195,000 subunit molecular weight so that our data and theirs may be compared. With the exception of half-cystine, our data and theirs are similar with about half the residues being within ±5%. It should be noted that in the present study, half-cystine was determined after performic acid oxidation which tends to prevent the under-

estimation of this residue. Since values for valine, iso-leucine, and leucine increase with time, these were determined after hydrolysis for 120 h.

Immunological studies. The results of these studies essentially confirmed those previously reported by Zimmerman et al (16). Human plasma which contained a high titer of a spontaneously formed inhibitor to factor VIII prolonged the partial thromboplastin time of the most purified fractions of normal factor VIII from 60 to 165 s; a buffer control was 155 s. Bands of identity were observed when absorbed rabbit antiserum to purified normal factor VIII was diffused against Step 1 normal factor VIII, as well as Step 1 hemophilic factor VIII. Functionally, when a 1:2 dilution of unabsorbed or absorbed antiserum to Step 2 normal factor VIII was incubated with normal human plasma at 37°C for 2 h, the effectiveness of the normal plasma in correcting the partial thromboplastin time of hemophilic plasma was markedly diminished as evidenced by a prolongation of the PTT from 79.2 s to 104.2 and 125.9 s, respectively.

Effect of thrombin on factor VIII. Fig. 6 shows the response of purified normal factor VIII to trace quantities of thrombin with time. Percent factor VIII activity was estimated by the one-stage partial thromboplastin time method. The value shown on the ordinate is the percent activity given by the purified factor VIII-thrombin mixture normalized to the activity given by purified factor VIII alone. Buffer, which contained the same final thrombin concentrations as did the factor VIII-thrombin mixture, gave clotting times that were not significantly different from those of buffer alone. As can be seen, final thrombin concentrations of 0.05 U/ml rapidly activated and then progressively inactivated factor VIII. Purified hemophilic factor VIII, however, activated only slightly in response to thrombin. The reduced SDS-gels in Fig. 6 show that neither activation or inactivation of factor VIII was accompanied by a change in subunit molecular weight. In this preparation two very faint bands of molecular weight approximately 100,000 and 130,000 were inconsistently observed; their intensity was not affected by exposure to thrombin. Experiments in progress indicate that these two bands, which can occasionally be found in trace amount in purified factor VIII, result from degradation of factor VIII by plasmin. In addition the intensity of the carbohydrate stain did not vary during activation and inactivation. Similarly, changes in carbohydrate staining intensity, as assessed by the periodic acid-Schiff reagent, were not detectable during incubation of hemophilic factor VIII with thrombin. Fig. 7 demonstrates the effect of different thrombin concentrations when incubated with a constant concentration of factor VIII for 1 min at 37°C. In this ex-
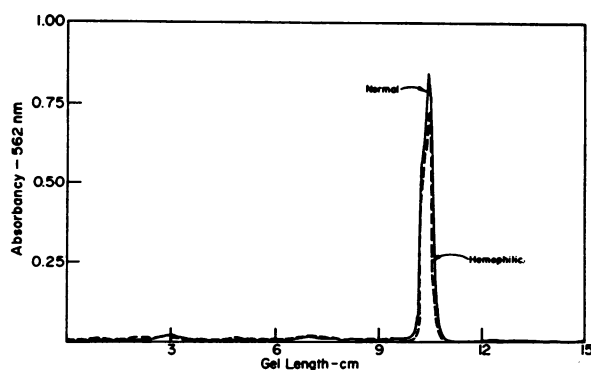
FIGURE 5 Gel scan of the reduced and carboxymethylated subunit from normal and hemophilic factor VIII after isoelectric focusing in polyacrylamide gels. The top of the gel is represented by the 0 point on the abscissa.

periment, factor VIII activity was estimated with the two-stage assay, especially modified so as to be sensitive to thrombin-induced activation. The first stage incubation time was held at 2 min in order to optimally demonstrate the thrombin effect on factor VIII. Were this time to have been prolonged the inactivation of factor VIII by thrombin as well as by plasmin (present in the serum components of this assay) (41) might have obscured the immediate activation of factor VIII by thrombin. The concentrations of thrombin used in this study did not affect the results of the two-stage assay when tested in buffer alone. Clearly at a final thrombin

TABLE III
Amino Acid Composition of Human Factor VIII*

Amino acid	Present study	Hershgold et al. (8)
Lysine	95.94	99.06
Histidine	53.04	33.74
Arginine	94.38	85.02
Aspartic acid	162.63	154.25
Threonine	99.65	99.06
Serine	129.87	122.85
Glutamic acid	224.06	201.24
Proline	117.00	101.60
Glycine	131.63	128.70
Alanine	96.92	105.89
Half-cystine	120.71‡	45.83
Valine	151.71§	116.22
Methionine	24.77	19.31
Isoleucine	62.21§	61.82
Leucine	134.36	133.38
Tyrosine	34.71	44.85
Phenylalanine	49.34	51.68

* Residues per 195,000 molecular weight subunit.
‡ Determined after oxidation.
§ 120 h hydrolysis.
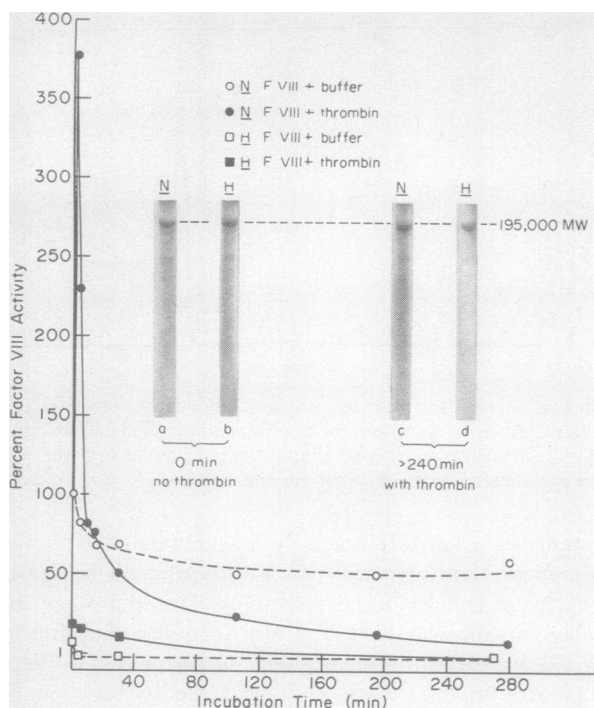
Subunit Structure of Human Factor VIII   2205

FIGURE 6 The effect of thrombin on the activity of puri-
fied normal and hemophilic factor VIII. Activity was as-
sessed by the one-stage activated partial thromboplastin
time assay. Gel a and gel b compare the reduced SDS-gel
electrophoretic patterns of normal and hemophilic factor
VIII before the addition of thrombin at 0 time. Gel b and
gel c compare the mobilities of reduced normal (N) and
hemophilic (H) factor VIII after each had been exposed
to 0.05 U/ml of thrombin > 240 min. No detectable change
in the subunit molecular weight (MW) of normal factor
VIII occurred despite activation and inactivation during this
time period. Similarly no apparent change in the molecular
weight of the hemophilic factor VIII subunit occurred
during exposure to thrombin.

concentration of about 0.03 U/ml, activation or factor
VIII occurred; however, at thrombin concentrations
above 0.312 U/ml, factor VIII activity was rapidly
destroyed.

DISCUSSION

The studies reported here expand previous observations
(1–11) concerning certain properties of normal, func-
tional human factor VIII, and in addition, support im-
munological data (13–18) indicating that nonfunctional
factor VIII is present in classical hemophilia. Several
investigators have shown that highly purified factor
VIII can be prepared by filtering factor VIII concen-
trates through columns of 2–4% agarose as the final
purification step (1–11). As confirmed in the present
study, this latter purification step results in an approxi-
mate 10,000-fold increase in specific activity with re-

spect to normal plasma. When glycine-precipitated fac-
tor VIII was the starting material, the yield of normal
factor VIII after the 4% agarose chromatography step
was about 29% as shown in Table I. When fresh-
frozen plasma was used, the yield of factor VIII after
agarose chromatography was somewhat lower, being
about 10% of the activity in the starting plasma; this
value is in good agreement with previously reported
recoveries (8, 10, 20). Moreover, the recovery of puri-
fied factor VIII from agarose columns was usually about
this latter value despite whether the starting material
was glycine-precipitated factor VIII, cryoprecipitate, or
fresh-frozen plasma. After rechromatography on aga-
rose gel columns, a single, symmetrical peak was ob-
served in the void volume region and no further puri-
fication was achieved.

In the case of DEAE-cellulose chromatography, how-
ever, the yield of purified factor VIII was much lower
—approximately 3.6% of the activity in the starting
plasma. Conceivably factor VIII prepared by this
method is partially degraded or modified with a re-
sulting loss of activity during the ion exchange chroma-
tography step. The important feature of the DEAE-
cellulose step, however, was the demonstration that
factor VIII activity coincided with the elution of a pro-
tein which, after reduction, was found to contain a
single subunit species identical in molecular weight to
the subunit species of factor VIII prepared by agarose
gel filtration; in addition both contained carbohydrate.

Since the yield of normal factor VIII appeared to be
consistently proportional to the activity in the starting
material and since Zimmerman et al. (16) observed
that hemophilic plasma contains normal amounts of
nonfunctional factor VIII-like antigen, the yield of void
volume protein and factor VIII activity was examined
and compared for six normal men and six classical
hemophiliacs. It should be noted that since yields of
factor VIII are calculated on the basis of activity, no
such calculations could be made for hemophilic plasmas.
It was assumed that if a normal level of nonfunctional,
abnormal factor VIII protein was present in hemophilic
plasmas, fractionation procedures identical to those used
for normal plasmas might result in the recovery of final
quantities of hemophilic factor VIII similar to those
of normal factor VIII. This indeed proved to be the
case. As shown in Fig. 2, when material prepared
from hemophilic plasma was chromatographed on 4%
agarose, a protein peak was observed in the void vol-
ume for each hemophilic preparation just as in the
case of each normal preparation. While the expected
amount of protein was observed, however, very little
factor VIII activity was found in this peak for the
hemophilic samples. The fact that some activity was
noted despite < 1–5% activity levels in the six hemo-

philic plasmas is not surprising in view of the concentration effect achieved by the purification method.

From the observation that factor VIII activity occurs in or very near the void volume during agarose chromatography, the native molecular weight of human factor VIII has been estimated at approximately 1–2 million (3, 8, 10). Hershgold et al. (8) reported an estimated $s_{25,w}$ of 16.3 in 6 M guanidine hydrochloride which tends to substantiate its large size and suggests that the native molecule is held together by covalent bonds. The polyacrylamide gel electrophoretic results of Kass, Ratnoff, and Leon (3) and Hershgold et al. (8) suggested that the factor VIII molecule was too large to enter even a 3% polyacrylamide gel. Our initial observations suggested this to be the case even after exposing factor VIII to denaturing solvents such as guanidine hydrochloride and sodium dodecyl sulfate (5). Since then Marchesi, Shulman, and Gralnick have reported similar findings (11), and more recently Schmer, Kirby, Teller, and Davie (10) have observed that bovine factor VIII does not enter a 3.25% polyacrylamide gel even in the presence of 8 M urea. These studies all indicated that any subunits in the factor VIII molecule must be joined by covalent bonds. When factor VIII was reduced by β-mercaptoethanol and examined by SDS-gel electrophoresis a single band was observed with a molecular weight initially estimated to be about 215,000 daltons. Since then the subunit molecular weight of rabbit myosin, an important standard in our SDS-gel electrophoretic analyses, has been more accurately determined (28) and we now report the molecular weight of the factor VIII subunit to be 195,000 (41). It is to be emphasized that on SDS-gels, the factor VIII subunit had a mobility which was consistently less than the myosin subunit of 194,000 molecular weight. As shown in Fig. 3, normal and hemophilic purified factor VIII had identical subunit molecular weights and moreover, both contained carbohydrate when stained by the periodic acid-Schiff reagent. To date, there is little consensus about the actual size of the human factor VIII subunit. While our finding of 195,000 molecular weight for the factor VIII subunit is smaller than the value of 240,000 molecular weight reported by Marchesi et al. (11), it is of interest that both values suggest that the human factor VIII molecule is composed of several very large subunit polypeptide chains. The differences between the presently reported molecular weight of 195,000 and the value of 240,000 obtained by Marchesi et al (11) can be explained by their use of non-reduced fibrinogen as a marker for the high molecular weight portion of the standard curve. Of concern are the completely divergent results of Hershgold et al. (8) which suggest that the human factor VIII molecule is composed of 22,000
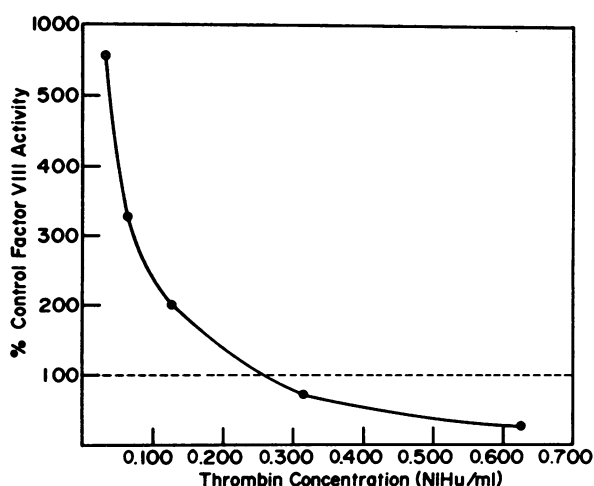

FIGURE 7 The immediate effect of thrombin concentration on the activation and inactivation of purified normal factor VIII. Factor VIII activity is expressed as a percentage of the purified factor VIII activity prior to the addition of thrombin.

molecular weight subunits held together by disulfide bonds. This latter value cannot be evaluated fully since details of the experiments have not yet been published. From their preliminary molecular weight determinations by sedimentation equilibrium analysis, Schmer et al. (10) have recently suggested that the subunits of bovine factor VIII may not be identical and instead, may range in size from 85,000 to 198,000. However, when their bovine factor VIII preparation was reduced and analyzed in an 8 M urea-3.25% polyacrylamide gel electrophoretic system, a single band of relatively low mobility was observed, thus suggesting that the bovine factor VIII subunit is indeed large and probably homogeneous in size. Moreover, the results of sedimentation equilibrium ultracentrifuge analyses in the present study indicate that the human factor VIII subunit is homogeneous by size, and has an approximate molecular weight of 202,000 which is very close to the value obtained by SDS-gel electrophoresis.

In electrophoretic systems in which mobility is primarily a function of charge rather than size, the patterns of purified factor VIII preparations have been difficult to interpret since a substantial amount of sample usually remained at the origin (3, 8). This is probably due to the large size of the molecule which prevents its penetrating the usual 5% polyacrylamide gels (5, 10, 11). In the present study non-reduced factor VIII did not penetrate even the most loosely cross-linked acrylamide gels (3.36%). When reduced and alkylated and the examined by isoelectric focusing, however, a single band, albeit slightly diffuse, was observed. A band of identical mobility was noted when reduced and alky-

lated purified hemophilic factor VIII was examined by isoelectric focusing. These latter observations are in accord with those reported by Schmer et al. (10) who found that reduced and alkylated bovine factor VIII migrated as a single spot in zone electrophoresis. When the results of SDS-gel electrophoresis, zone electrophoresis and isoelectric focusing are combined, it appears that the repetitive subunits in human or bovine factor VIII are identical in size and charge. This raises doubt concerning sedimentation equilibrium data which suggest the molecular weights of bovine factor VIII subunits vary from 85,000 to 198,000 (10) since molecular weight differences of this magnitude should be accompanied by easily detectable charge differences on zone electrophoresis or isoelectric focusing.

The amino acid composition data suggest that the aromatic residue content of normal human factor VIII is lower than usually observed for most proteins; Schmer et al. have reported similar findings for bovine factor VIII (10). These data provide a likely explanation for the past observations of poor absorption at 280 nm for factor VIII preparations. Table III compares the amino acid composition of the human factor VIII subunit from the present study with that calculated from the data of Hershgold et al (8) on the basis of a subunit molecular weight of 195,000. As can be seen, the compositions are fairly similar except for marked discrepancies in the histidine values and half-cystine content. The latter variation can be explained since in the present study half cystine was determined after performic acid oxidation and therefore can be considered the more reliable value. We have no explanation for the discrepant histidine values. The high degree of similarity between the amino acid compositions in the present study and those reported by Hershgold et al. (8) suggest that the same protein was being examined. Because of the obvious difficulty in obtaining adequate quantities of hemophilic plasma, purified hemophilic factor VIII could not be prepared in amounts sufficient for determination of amino acid composition.

Our immunological studies largely confirm those reported by others (13–18). A spontaneously formed human factor VIII inhibitor as well as antiserum prepared in rabbits to highly purified human factor VIII clearly neutralized factor VIII activity in specific factor VIII assays. Moreover, identical precipitin bands were noted when the antiserum was tested against purified normal or hemophilic factor VIII by immunodiffusion.

While other workers have reported that factor VIII can be activated and subsequently inactivated by trace amounts of thrombin (41–46), the present study represents the first reported attempt to determine if changes in factor VIII structure occur concomitantly with changes in function. In this study, no detectable change in subunit molecular weight or change in intensity of protein or carbohydrate staining was discernible with activation and inactivation of normal factor VIII by thrombin. Similar treatment of purified factor VIII from hemophilic plasmas demonstrated only a slight increase in activity with respect to protein concentration and no detectable structural changes. Because of the well-documented proteolytic activity of thrombin toward fibrinogen (47) and fibrin stabilizing factor (48), it seems likely that catalytic amounts of thrombin affect factor VIII activity by similar specific proteolytic modification of the factor VIII subunits. This hypothesis receives additional support from the observation that trypsin, a known protease of lesser specificity, is also capable of activating and inactivating factor VIII without initial structural change in the factor VIII subunit; extensive degradation, however, occurs following prolonged incubation (41). These observations suggest that both activation and inactivation of normal factor VIII may occur by cleavage of peptide bonds resulting in fragments, the sum of which molecular weights is less than about 10,000, since the limits of resolution of the SDS-gel system are about ± 5%. It seems probable then, that thrombin produces an initial increase in factor VIII activity by cleaving a very small peptide from the factor VIII molecule. The subsequent complete loss of factor VIII activity may then result from additional cleavage of small peptide(s) by thrombin.

In conclusion the results of the present study indicate the following: (a) human factor VIII is a plasma glycoprotein with a molecular weight of 1–2 million; it is composed of 195,000 molecular weight repetitive subunits, most likely totaling 5–10, joined by disulfide bonds; (b) hemophilic factor VIII appears identical to normal factor VIII in subunit molecular weight, carbohydrate content, electrical charge, and antigenic determinants; (c) the activation and inactivation of normal factor VIII by thrombin without an apparent change in molecular weight and without the appearance of additional peptide bands suggest that the release of peptides totaling less than 10,000 molecular weight per factor VIII subunit may account for both events.

ACKNOWLEDGMENTS

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