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Purification and characterization of a highly purified human factor VIII consisting of a single type of polypeptide chain

(blood coagulation/factor VIII antigen/factor X_a/protein C/thrombin)

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ABSTRACT **Human factor VIII was purified 350,000-fold (relative to plasma) from a commercial factor VIII concentrate. The procedure used standard protein separation techniques and was performed in the absence of protease inhibitors. The product has a specific activity of 4,900 units/mg, an activity-to-antigen ratio of 75:1 (unit/unit) and no more than 0.1% von Willebrand protein. Electrophoresis of the reduced protein in a denaturing polyacrylamide gel showed a single major band of M_r 100,000. Procoagulant activity was eluted from a nondenaturing gel after electrophoresis in the region of the single major band. Thrombin converted the M_r 100,000 polypeptide to a polypeptide of M_r 75,000. The procoagulant activity was increased 10-fold by thrombin or factor X_a and was completely inhibited by activated protein C or factor VIII inhibitor plasma. This factor VIII preparation consists of a single high molecular weight polypeptide chain and has the highest specific activity thus far reported for human factor VIII.**

The most common inherited bleeding disorders are associated with deficiencies or defects in factor VIII or von Willebrand protein. Much of the physiological role and structural features of the von Willebrand protein have been characterized (1); much less is known about factor VIII. Two major factors have hindered the study of factor VIII—the low concentration of factor VIII in plasma, and the difficulty of obtaining a purified, homogeneous preparation. Recent reports have described a high degree of purification and some characterization of bovine factor VIII (2), porcine factor VIII (3), and human factor VIII (4). However, the final preparations contained several polypeptide chains when analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions.

We report here a procedure for purification of human factor VIII from plasma which utilizes calcium dissociation and differential size and charge chromatography. The purified factor VIII is obtained in reasonable yield, is functionally potent, consists of a single major polypeptide chain, and contains less than 1% von Willebrand protein.

METHODS

Materials. Commercial factor VIII concentrate (Koate) was kindly provided by Cutter Laboratories (Berkeley, CA). Human factor X was prepared from commercial concentrate (Konyne; Cutter Laboratories) by the method of Miletich *et al.* (5). The purified factor X was activated with Russell's viper venom (Sigma) according to the method of DiScipio *et al.* (6) and purified on DEAE-cellulose. Human thrombin (1,650 units/mg) was kindly provided by John Fenton (Albany, NY) and activated bovine protein C (220 μg/ml) was kindly provided by Fred Walker (Terre Haute, IN). The anti-factor VIII antibody, supplied as Fab' was generously provided by Howard Reisner (Chapel Hill, NC) and was labeled with ^{125}I by the method of Helmkamp *et al.* (7). Antibody to von Willebrand protein was made by the method of Zimmerman *et al.* (8). The source of von Willebrand protein used for the enzyme-linked immunosorbant assay was the void volume fraction eluted from Sepharose CL-4B obtained during the purification of factor VIII. Hemophiliac plasma was obtained from a patient with severe factor VIII deficiency as shown by a glass clotting time >60 min (9). The factor VIII inhibitor plasma used in this study had a titer of 6,400 Bethesda units. BioGel A-15m, Sepharose CL-4B, and QAE cellulose (fine mesh) were purchased from Bio-Rad Laboratories, Pharmacia, and Sigma, respectively. The protein silver staining kit was purchased from Bio-Rad.

Assays. Protein concentration was determined by absorbance at 280 nm corrected for light scattering; an $E^{1\%}_{280}$ corrected of 10.0 for factor VIII was assumed. Factor VIII procoagulant activity was quantitated by a one-stage clotting assay (10). In the presence of 0.25 M $CaCl_2$, factor VIII procoagulant activity was measured by using a modified one-stage assay (11). Factor VIII antigen was measured according to the procedure of Reisner *et al.* (12); 1 unit of factor VIII antigen represented that amount present in 1 ml of pooled normal human plasma. Inhibition of factor VIII procoagulant activity by inhibitor plasma was measured by mixing 1 vol of sample containing factor VIII with 1 vol of human plasma containing inhibitor to factor VIII, incubating for 5–10 min, and then measuring residual factor VIII as described above. The amount of von Willebrand protein in the purified factor VIII protein was determined by an enzyme-linked immunosorbant assay (13).

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed according to O'Farrell (14) on a 4.5% stacking gel with a 6% separating gel. Electrophoresis was at 25 mA for 4 hr. Nondenaturing polyacrylamide gel electrophoresis was on a 5% gel in 25 mM Tris/192 mM glycine, pH 8.3. Electrophoresis was at 250 V for 3 hr at 4°C. Gels were stained for protein by using silver nitrate (15). Protein was eluted by slicing the unstained gel at 5-mm intervals and adding each slice to 0.3 ml of 50 mM Tris·HCl, pH 7.0/150 mM NaCl containing 200 μg of ovalbumin per ml. The mixture was incubated at 4°C for 2–3 hr and assayed for factor VIII procoagulant activity as described above.

Purification of Factor VIII. All steps were performed at room temperature unless otherwise indicated, and all buffers contained 0.02% sodium azide. To prepare Koate, fresh-frozen citrated human plasma (collected by plasmapheresis) is subjected to a proprietary process of cryoprecipitation, the redissolved precipitate is treated to remove vitamin K-dependent

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Table 1. Purification of human factor VIII

	Volume, ml	Total protein, mg	Total activity, units	Specific activity, units/mg	Yield, %	Purification, -fold
Plasma	—	—	—	0.014	—	1
Koate	250	12,580	11,000	0.87	100	62
Bio-Gel A-15m; ammonium sulfate; dialysis	22	318	9,469	29.8	86	2,126
Ca^{2+} dissociation; Sepharose CL-4B; dialysis	110	13.2	6,850	519	62	37,067
QAE cellulose	29.5	0.28	1,396	4,986	12.7	356,122

All values represent the mean of two preparations.

proteins, pH and temperature are adjusted to purify the adsorbed cryoprecipitate further to a specific activity of 1 unit of factor VIII per mg of protein, and the final solution is freeze-dried. Ten bottles of this commercial factor VIII concentrate were reconstituted in a final volume of 50 ml of 5 mM sodium citrate/1 mM $CaCl_2$/135 mM NaCl/5% (wt/vol) dextrose, pH 7.35, and applied to a Bio-Gel A-15m column (5 × 100 cm) equilibrated in the above buffer. The flow rate was maintained at 200 ml/hr, and 25-ml fractions were collected. Fractions containing factor VIII procoagulant activity eluted in the excluded column volume and were pooled and concentrated with ammonium sulfate (40% saturation). The resulting pellet was stored at −70°C.

This procedure was repeated, and the following steps used material derived from 50 bottles of the processed commercial concentrate. The ammonium sulfate precipitates were thawed at 4°C and resuspended in 10–15 ml of 50 mM imidazole·HCl, pH 7.0/150 mM NaCl (buffer A). This material was dialyzed against the same buffer for 18–24 hr (three changes). The protein sample was made 250 mM in $CaCl_2$ by adding 1/9th vol of a 2.5 M solution prior to chromatography on Sepharose CL-4B (2 × 90 cm) equilibrated in buffer A containing 250 mM $CaCl_2$. Because of the volume of sample, it was necessary to perform this step in two successive column runs, with less than 14 ml applied per run.

Fractions with procoagulant activity were pooled and dialyzed overnight in 6 liters of buffer A. The protein sample was applied to a 20-ml column of QAE cellulose equilibrated in buffer A. The resin was washed with buffer A until no protein could be detected in the eluant by absorbance at 280 nm. The resin was washed with 40 ml of 50 mM imidazole·HCl, pH 7.0/200 mM NaCl, and then eluted with a linear gradient of 0.2–1 M NaCl (90 ml of each). Fractions with factor VIII procoagulant activity were pooled. A portion was dialyzed against 200 mM ammonium bicarbonate (pH 7.5), frozen in a dry ice/ethanol bath, and lyophilized. To the remainder was added ovalbumin (100 μg/ml) and $CaCl_2$ (5 mM) to aid in preserving the procoagulant activity. This material retained about 30% of its original activity after 2 weeks when stored at 0°C.

RESULTS

Purification of Human Factor VIII. Factor VIII was purified about 6,000-fold over the starting material, commercial factor VIII concentrate, with a final yield of 13%, representing a purification of approximately 350,000-fold over plasma (Table 1). Factor VIII antigen and procoagulant activities were monitored throughout the purification procedure and were substantially separated from each other in the latter stages of preparation. Gel filtration of calcium-dissociated factor VIII on Sepharose CL-4B resulted in a small peak with procoagulant activity at the void volume and a single major procoagulant peak which eluted within the included column volume (Fig. 1). However, at least two peaks of factor VIII antigen were eluted, one within the void volume and the other(s) within the included volume. The last peak appeared to have a leading shoulder which corresponded closely to the peak of factor VIII procoagulant activity. The major factor VIII peak obtained from the Sepharose CL-4B column was chromatographed on QAE cellulose (Fig. 2). Although the bulk of the procoagulant activity eluted between 0.27 and 0.37 M NaCl, the factor VIII antigen eluted in multiple peaks at 0.2, 0.26, and 0.47 M NaCl. The ratio of factor VIII

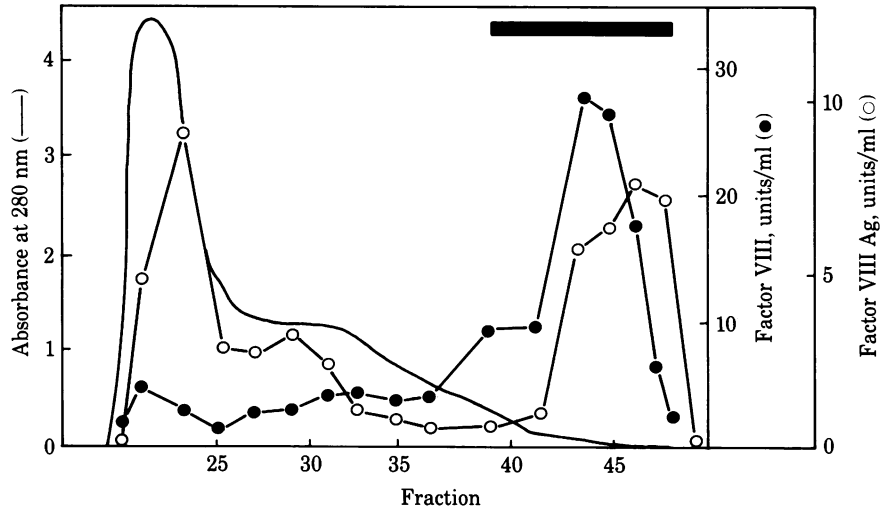

FIG. 1. Chromatography of factor VIII on Sepharose CL-4B in 0.25 M $CaCl_2$. Starting material obtained from the initial separation through Bio-Gel A-15m was adjusted to 0.25 M in $CaCl_2$ and applied to a Sepharose CL-4B column (2 × 90 cm) equilibrated in buffer A containing 0.25 M $CaCl_2$. The flow rate was 57 ml/hr, and 5.5-ml fractions were collected. The solid bar indicates the fractions pooled for subsequent purification on QAE cellulose. "Factor VIII" refers to procoagulant activity; "Factor VIII Ag" refers to factor VIII antigen.

procoagulant activity to antigen in the final preparation (solid bar, Fig. 2) was 75:1 (unit/unit; average of two preparations). Enzyme-linked immunosorbant assay showed that the purified protein contained 0.1% von Willebrand protein.

Electrophoretic Analyses. The purified factor VIII protein migrated as a single major band of M_r 100,000 in a reduced sodium dodecyl sulfate/6% polyacrylamide gel (Fig. 3 *Left*, lane C). This preparation also contained traces of material with M_r >220,000. Staining with Coomassie blue revealed only a single band of M_r 100,000 at a loading of 12 μg of protein (not shown). The unreduced protein migrated as a single band with apparent M_r 72,000 (Fig. 3 *Right*). Analysis of this preparation by electrophoresis in a sodium dodecyl sulfate/10% polyacrylamide gel showed no low molecular weight protein (results not shown).

Recovery of Procoagulant Activity from Polyacrylamide Gels After Electrophoresis. In order to confirm that the procoagulant activity was associated with the major protein, electrophoresis in polyacrylamide was performed under nondenaturing conditions. One portion of the gel was stained for protein (Fig. 4 *Upper*) and a second portion was cut into slices, incubated in buffer, and assayed for factor VIII procoagulant activity (Fig. 4 *Lower*). Those fractions with detectable activity (about 3% of the total applied activity) corresponded to the area of the gel containing the major broad protein band. Some activity was associated with protein that failed to enter the gel; the reason for this is not apparent.

Characterization of Factor VIII. The procoagulant activity of the purified factor VIII was increased about 10-fold by human thrombin (Fig. 5 *Upper*, solid circles). Little or no increase in procoagulant activity was observed with thrombin concentrations <1 μg/ml (data not shown). When purified protein was treated with thrombin (10 μg/ml) and analyzed by electrophoresis on a reduced denaturing gel, the M_r 100,000 band was converted completely to a band of M_r 75,000 (Fig. 3 *Left*, lane D). The trace polypeptides with M_r >220,000 were unaltered by treatment with thrombin. Stoichiometric amounts of thrombin (at least the molar equivalent based upon M_r 100,000 for factor VIII) were required for complete conversion to the M_r 75,000 form; use of a lower level of thrombin (1 μg/ml) resulted in conversion of only a small proportion of the material to the lower molecular weight band (Fig. 3 *Left*, lane E). The extent

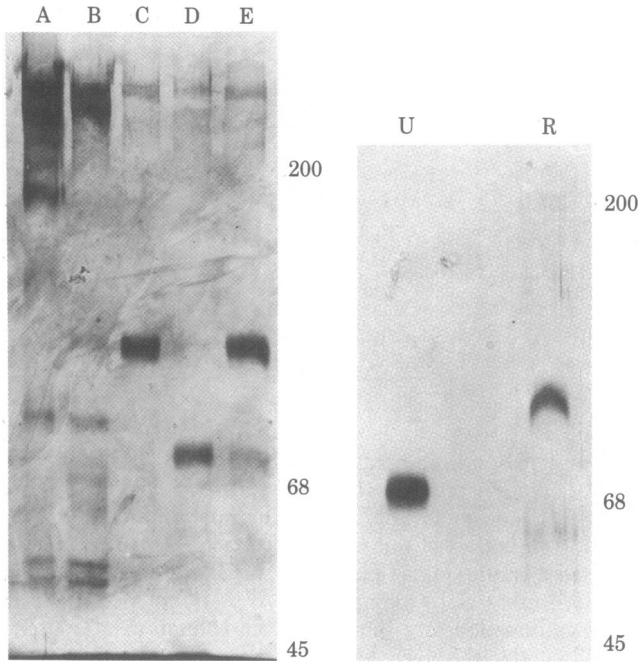

FIG. 3. Sodium dodecyl sulfate/6% polyacrylamide gel electrophoresis of factor VIII. The gels were stained with silver nitrate. (*Left*) Lanes: A, factor VIII pool after Bio-Gel A-15m column chromatography; B, factor VIII pool after $CaCl_2$ dissociation and Sepharose CL-4B column chromatography (Fig. 1); C, factor VIII pool after QAE cellulose column chromatography (Fig. 2); D and E, factor VIII obtained after elution from QAE cellulose and treated with thrombin (10 and 1 μg/ml, respectively) for 20 min at 23°C prior to electrophoresis. (*Right*) Reduced (R) and unreduced (U) factor VIII. The M_r values of the protein standards are expressed as 10^{-3}.

of conversion appeared to parallel the activation of factor VIII as measured by the clotting assay.

Factor X_a in the presence of phospholipid (Fig. 5 *Upper*) increased the factor VIII procoagulant activity about 10-fold; this enhanced activity appeared to be more stable than that produced by thrombin. The effect of activated bovine protein C, a serine protease with inhibitory activity against factors V and

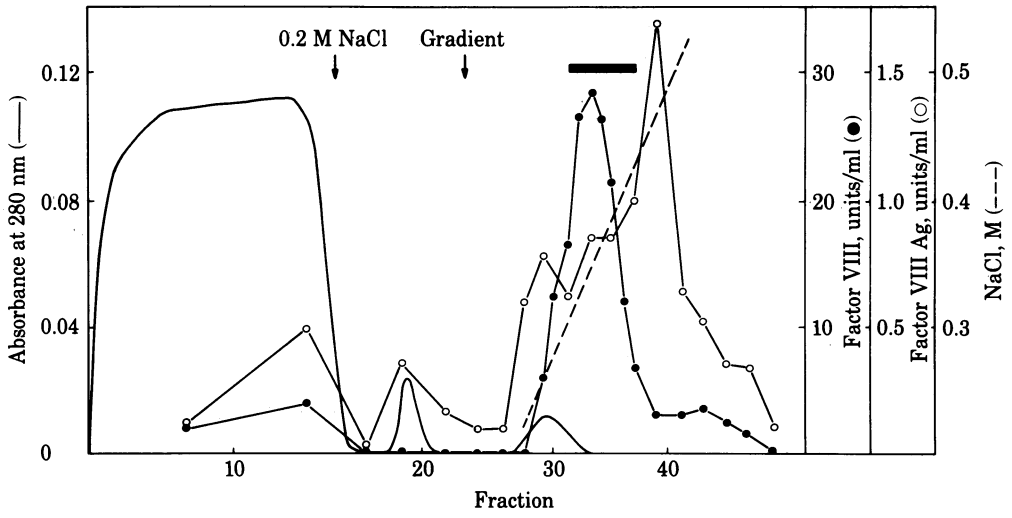

FIG. 2. Chromatography, on QAE cellulose, of partially purified factor VIII eluted from Sepharose CL-4B. The 20-ml polypropylene column of QAE cellulose was equilibrated in buffer A, and the nonadsorbed protein was washed from the column with buffer A. This was followed by a stepwise elution (40 ml) with 50 mM imidazole·HCl, pH 7.0/0.2 M NaCl and a linear NaCl gradient, in the same buffer, of 0.2–1 M (90 ml of each). Ten-milliliter fractions were collected through the 0.2 M elution and 5.5-ml fractions were collected during the linear gradient elution. The flow rate was 17 ml/hr.

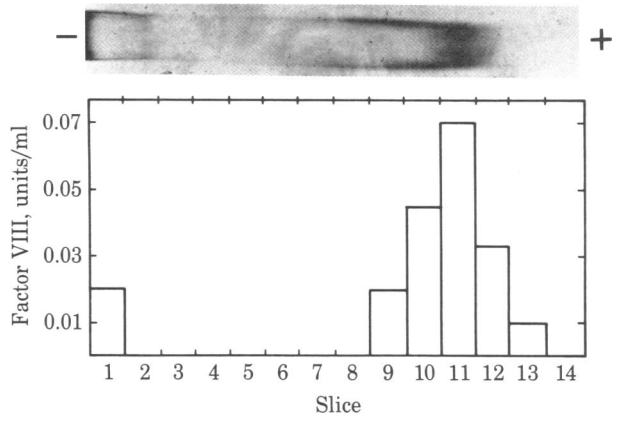

FIG. 4. Nondenaturing 5% polyacrylamide gel electrophoresis and elution of factor VIII procoagulant activity. Approximately 3.4 units (0.65 μg) of procoagulant activity was applied to two parallel lanes and electrophoresed; then, one lane was stained with silver nitrate for protein (*Upper*). The other lane was sliced into 5-mm segments and the protein was eluted and assayed for procoagulant activity (*Lower*). About 2–4% of the applied activity was recovered from the gel slices.

VIII (2), is shown in Fig. 5 *Lower*. The factor VIII procoagulant activity was inactivated quickly, in comparison with the activity of the control sample.

Additionally, factor VIII procoagulant activity could be blocked by a factor VIII inhibitor from a patient with classical hemophilia. When mixed with a severalfold excess (unit/unit) of factor VIII inhibitor, >95% of the factor VIII procoagulant activity was immediately abolished (data not shown).

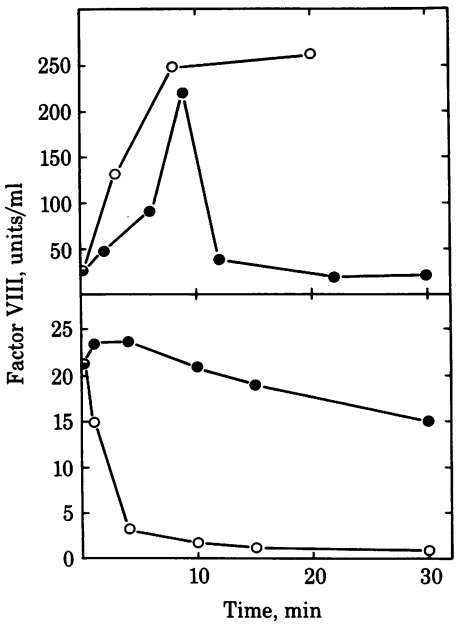

FIG. 5. Changes in factor VIII procoagulant activity after reaction with selected enzymes at 23°C. (*Upper*) Time course of activation and inactivation of procoagulant activity by thrombin (●) and factor X_a (○). The reaction mixtures contained 25 units of procoagulant activity per ml (5.5 μg/ml), 25 mM imidazole·HCl at pH 7.0, 150 mM NaCl, and thrombin (16 units/ml, 10 μg/ml) or factor X_a (1 μg/ml). The reaction mixture with factor X_a contained, in addition, inositol (100 μg/ml) and 5 mM $CaCl_2$. (*Lower*) Time course of inactivation of factor VIII procoagulant activity by activated protein C (○) compared to a control without activated protein C (●). The reaction mixture contained 25 units of procoagulant activity per ml (5.5 μg/ml), 25 mM imidazole·HCl at pH 7.0, 150 mM NaCl, and inositol (100 μg/ml), with or without activated protein C (440 ng/ml).

The purified factor VIII had no effect in a nonactivated, partial thromboplastin time assay, indicating that the preparation did not contain activated clotting factors that circumvented the factor VIII-dependent step in the clotting cascade.

DISCUSSION

Human factor VIII has been purified from plasma approximately 350,000-fold (to 4,900 units/mg) with an overall yield of 13%. The purified protein is stable, with respect to activity and appearance in sodium dodecyl sulfate/polyacrylamide gel electrophoresis, for at least 2 weeks when stored unfrozen at 0°C in the presence of carrier protein. The purification procedure used a combination of standard protein separation techniques and was performed in the absence of protease inhibitors. The stability of the final product is particularly noteworthy and may be due to the absence of proteases in the starting material.

Analysis of the final preparation by electrophoresis in a reduced sodium dodecyl sulfate/polyacrylamide gel showed a single major band with M_r 100,000. The evidence that this material is factor VIII is as follows: (*i*) recovery of factor VIII procoagulant activity from non-denaturing gels in the same position as the major protein band; (*ii*) potentiation of procoagulant activity by thrombin and factor X_a; (*iii*) inactivation by longer exposure to thrombin, by activated protein C, and by spontaneous inhibitors against factor VIII; and (*iv*) conversion by thrombin of the M_r 100,000 polypeptide to M_r 75,000. The trace material of M_r >220,000 is probably not factor VIII because it was unaffected by treatment with thrombin.

The preparation contained a trace amount (0.1%) of material that reacted in an immunoassay using a polyclonal antibody against von Willebrand protein. Because the anti-von Willebrand antiserum probably contains a small amount of non-neutralizing antibody against factor VIII, and perhaps other antigens, the trace amount of protein detected in this assay could be von Willebrand protein, factor VIII reacting with traces of anti-factor VIII antibody, or another, as yet unidentified, contaminant.

Preliminary experiments using conventional Sepharose gel filtration, high performance liquid chromatography, and electrophoresis in nondenaturing polyacrylamide gradient gels (not shown here) suggest that the factor VIII may exist as a noncovalent complex of two to four M_r 100,000 subunits. More accurate determination of molecular weights will require studies by sedimentation equilibrium, but our results are consistent with those of Weinstein *et al.* (16) who showed that the major component of factor VIII antigen in normal human plasma has M_r 270,000. The subunit described here contains intrachain disulfide bonds because the apparent molecular size as determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the unreduced protein was less than that of the reduced form.

Two other properties of our factor VIII preparation are noteworthy. First, the concentration of thrombin required to activate the factor VIII procoagulant activity and to cleave the M_r 100,000 polypeptide was stoichiometric, as noted by others (3, 17). We have not yet identified a polypeptide chain of M_r ≈25,000 which would account for the change in M_r following cleavage by thrombin.

Second, the purified factor VIII retained a small amount of factor VIII antigen. This could be explained by activation of factor VIII during the purification. However, it is unlikely because the purified material is stable during storage and can be activated by thrombin and factor X_a, and most of the antigen can be separated chromatographically from procoagulant activity. A comparable separation of these two activities has been noted (18), and experiments in which factor VIII was fraction-

ated by using sucrose density gradients and gel filtration columns show that the elution profiles of factor VIII and factor VIII antigen do not always coincide with one another (19). The explanation for this separation of factor VIII from factor VIII antigen is not known, but it may be due to heterogeneity of either the antibody or the antigen or both; the latter is demonstrated clearly by the multiple elution peaks from QAE cellulose columns.

Fulcher and Zimmerman (4) recently purified human factor VIII approximately 164,000-fold; when analyzed by sodium dodecyl sulfate/polyacrylamide gel, this preparation consisted of a major doublet with M_r 79,000 and 80,000 and at least six additional faintly staining polypeptides with larger M_r. After treatment with thrombin, the higher M_r polypeptides apparently disappeared, and new bands appeared with M_r 71,000, 72,000, and 54,000. Factor VIII also has been purified to a similar specific activity from bovine and porcine plasma. The bovine protein contained three polypeptides, with equal staining intensities, of M_r 93,000, 88,000, and 85,000 (2). The porcine preparation contained three polypeptides of M_r 166,000, 130,000, and 76,000, the smallest of which possessed the most intense staining (3). All of the bovine chains were converted by thrombin to give a major doublet of M_r 69,000 and 73,000 and minor chains of M_r 55,000 and 38,000, whereas only the two higher M_r porcine chains were cleaved to give a major chain at M_r 67,000 and a minor one at 50,000. The multiplicity of polypeptides in these preparations may be the result of species differences or of proteolytic degradation. We cannot rule out the possibility that our material is an active fragment of a higher M_r factor VIII molecule.

Based on the reports of factor VIII purification from human, porcine, and bovine plasma, the plasma concentration of factor VIII is very low (approximately 200 ng/ml; 1.0 nM, on the basis of an oligomer M_r of 200,000). This is a much lower concentration than that of factor V or of the serine proteases with which factor VIII is supposed to interact. If this concentration of factor VIII proves to be correct, it will need to be taken into account in theories explaining the mode of action of factor VIII.

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