Immunologic Studies on Human Factor VIII (Anti-Hemophilic Factor A, AHF) Components Produced by Low-Ionic-Strength Dialysis

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Since dialysis of human factor VIII against buffers of low ionic strength yielded two distinct components, and since the factor VIII fraction isolated from normal plasma showed von Willebrand factor activity as defined by the corrective effect on abnormal platelet retention and ristocetin aggregation in von Willebrand's disease, the present studies were performed to determine if the correcting activities could be attributed to one or both of the components. Dialysis of factor VIII against buffers of low ionic strength led, however, to a decrease in factor VIII procoagulant activity and the reduction of the correcting activities, which suggested that the intact aggregate was required for procoagulant activity and for von Willebrand factor activity. In this respect dialysis of factor VIII at low ionic strength differed from dissociation at high salt concentrations. The two low ionic strength components were identified by the use of a rabbit antiserum against factor VIII, and could be distinguished on the basis of specific antigenic structures. Dialysis of factor VIII at low ionic strength led to a decrease in antigenic determinants closely related to factor VIII function. Specific antibodies to the low ionic strength components inhibited factor VIII activity in normal plasma, but the residual factor VIII activity was higher than that after inhibition with antibodies against intact factor VIII. Both antibodies interfered with von Willebrand factor activity.

Recent studies have indicated that human factor VIII (anti-hemophilic factor A, AHF) behaved as an aggregating series of homologous oligomers and that the oligomers were complexes of two different proteins. These proteins were demonstrated with polyacrylamide gel electrophoresis and cross-immuno-electrophoresis after dialysis of factor VIII against buffers of low ionic strength. The low-ionic-strength components were identified with a rabbit antiserum against factor VIII. This antiserum did not produce precipitates with plasma of patients with severe von Willebrand's disease (VWD) when tested by immunodiffusion, but did contain precipitating antibodies against both low-ionic-strength components.

The result of dialysis of factor VIII at low ionic strength differs from that of dissociation at relatively high ionic strength which yields a low-molecular-weight subunit (LMW) with factor VIII activity and a high-molecular-weight subunit (HMW) with von Willebrand factor activity. However, dialysis of factor VIII at low ionic strength results not only in a reduction of factor VIII procoagulant activity, but also as reported in this paper, in a decrease of von...
Willebrand factor activity. The von Willebrand factor activity is defined as the corrective effect on abnormal platelet retention and ristocetin aggregation in von Willebrand's disease.

The present paper reports the further immunologic characterization of factor VIII and its low-ionic-strength components. The experiments were designed to elucidate the structural and functional relationships between these components and the native factor VIII molecule. Specific antisera against the low-ionic-strength components were prepared, and the inhibitory effect of these antisera on factor VIII and von Willebrand factor activity was investigated. Circulating human inhibitors of factor VIII and rabbit antifactor VIII sera were absorbed with the two components to find out whether one or both of the components would block the factor VIII-neutralizing properties of these antisera.

METHODS

Purification of Factor VIII

Factor VIII was purified according to van Mourik et al. Reprecipitated human cryoprecipitate was used as starting material and subjected to gel chromatography on Sepharose 6B in the presence of dextran. Factor VIII was eluted at the void volume, together with the activity correcting the abnormal platelet retention in VWD.

Dialysis of Factor VIII at Low Ionic Strength

Factor VIII was dialyzed at low ionic strength (for about 24 hr) against 0.01 M sodium phosphate (pH 7.0) at room temperature. The effect of dialysis on factor VIII was monitored by polyacrylamide gel electrophoresis and cross-immunoelectrophoresis in agarose gel. The two low-ionic-strength components were identified as a slow-moving and a fast-moving component on the basis of their electrophoretic mobility.

Preparations of the slow- and fast-moving components were prepared as follows. Factor VIII was first dialyzed at low ionic strength against 0.01 M sodium phosphate until completion at room temperature, followed by fractional ammonium sulfate precipitation. The slow-moving component was precipitated by dialysis overnight at 4°C against 35% saturated ammonium sulfate, after which the fast-moving component was recovered at 50% saturation. Both components were dissolved in 0.04 M sodium phosphate (pH 8.5) and solutions of about 1 mg/ml were used.

Preparation of Antisera

Antifactor VIII was prepared as described elsewhere. Antisera against the fast- and slow-moving components were prepared by immunization of rabbits with equal volumes of crushed slices of the polyacrylamide gels containing the appropriate component (total protein concentration about 50 μg), together with Freund's complete adjuvant (Difco, Detroit). The fractions were localized by comparison with stained parallel runs. The rabbits were boosted 3 or 4 times at 2-wk intervals with crushed slices lacking Freund's adjuvant. Two weeks after the final booster, the rabbits were bled by heart puncture. The sera were absorbed with ethanol fractions of artificially prepared factor VIII-deficient plasma according to Zimmerman. The antisera were made specific for the slow- and fast-moving components by absorption with fractions containing the appropriate partially purified component obtained by gel chromatography of factor VIII on Sepharose 6B after dialysis at low ionic strength. More recently, the antisera were prepared and absorbed with fractions of slow- and fast-moving components, separated by ammonium sulfate precipitation. When tested by agarose gel immunodiffusion, the antisera showed one precipitin line against normal plasma and no line against plasma of patients with severe VWD.

The titer of the precipitating antibodies was compared with that of the titers found in antifactor VIII by cross-immunoelectrophoresis of factor VIII dialyzed at low ionic strength. The
FACTOR VIII

results (see Fig. 3) indicated that the titer of the anti-slow-moving component was comparable to the titer of the corresponding antibody in antifactor VIII, whereas anti-fast-moving component was about two times stronger.

Immunologic Techniques

Immunodiffusion in agarose gel and cross-immunoelectrophoresis in agarose gel were performed as described previously. Insolubilization of Antisera:

γ-globulin fractions of the antisera against the low-ionic-strength components were precipitated with Na₂SO₄ (18% w/v) and purified on Sephadex-DEAE A50 (Pharmacia, Uppsala, Sweden). Purified γ-globulin was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to Axen, Porath, and Ernbach(13 mg protein/g Sepharose), after which the gel was resuspended in 0.1 M Tris HCl buffer (pH 7.5) containing 5.1 mmole sodium citrate and packed in a chromatography column (K 15/30, Pharmacia). The columns were equilibrated overnight at 4°C with 0.1 M Tris HCl (pH 7.5) containing 5.1 mmole sodium citrate. Normal plasma was applied to the column and eluted at a speed of 3 ml/hr. To remove aspecifically adsorbed protein, 0.1 M Tris HCl (pH 7.5) containing 5.1 mmole sodium citrate was then applied until no protein could be detected in the effluent. The antigen-antibody complex was dissociated with 20–30 ml 3 M NaCNS (elution speed: 3 ml/hr). The columns were re-equilibrated with 0.1 M Tris HCl (pH 7.5) containing 5.1 mmole sodium citrate and could be used again.

Factor VIII Activity Determination

Factor VIII activity was determined with a one-stage assay, using diluted hemophilic plasma as reagent and Hyland standard as reference. To evaluate the inactivation of factor VIII activity in normal plasma by rabbit antisera against the slow- or fast-moving component, an equal volume of normal plasma was incubated for 1 hr at 37°C with the antibody diluted in normal rabbit serum, the residual factor VIII activity being expressed as a percentage of the activity in the control experiment in which normal rabbit serum was added instead of the antibody. The normal rabbit sera and the antibodies used had been inactivated for 1 hr at 56°C.

Antibody neutralization by the low-ionic-strength components was tested by incubation of rabbit antifactor VIII or human factor VIII inhibitors for 1 hr at 37°C with one or both of the low-ionic strength components, followed by a second incubation for 18 hr at 4°C. The test mixture was then centrifuged at 4°C for 30 min at 6000 g. The supernatant was inactivated for 1 hr at 56°C. The inhibitory capacity of the remaining antibody was then tested by mixing the supernatant with an equal volume of normal plasma. After an additional incubation period of 1 hr at 37°C, the residual factor VIII activity was determined and expressed as the percentage of the activity in the control experiment in which inactivated normal rabbit serum or normal human serum was added instead of rabbit antibody or human antibody, respectively. To investigate the influence of dilution on the factor VIII-inactivating property of the antibodies, the antibodies were incubated with saline instead of the components. Immunodiffusion experiments were performed to test the supernatants for antibodies remaining after absorption.

Platelet Function Tests

Von Willebrand factor activity of factor VIII before and after dialysis against buffers of low ionic strength was evaluated on the basis of the ability to correct both the abnormal platelet retention (as described elsewhere) and the abnormal ristocetin (Lundbeck, Copenhagen) aggregation in VWD. Ristocetin aggregation was performed in a Vitatron aggregometer (Meyvis, The Netherlands) by adding 0.2 ml of the factor VIII preparation or saline to 1.8 ml of a platelet rich plasma (PRP). The final platelet count was 200,000/µl PRP. After 3 min of incubation at 37°C ristocetin (2 mg/ml final concentration) was added and the mixture stirred at 1500 rpm. The initial velocity of aggregation was expressed calculating the tangent drawn to the steepest part of the aggregation curve on a potentiometric chart recorder. The initial velocities obtained with factor VIII dialyzed at low ionic strength were expressed as percentages of the initial velocity obtained.
Table 1. Effect of Dialysis (About 24 hr) at Low Ionic Strength on the Biologic Activities of Factor VIII

<table>
<thead>
<tr>
<th>Dialysis Buffer 0.01 M Sodium Phosphate (pH 7.0)</th>
<th>Factor VIII Activity (%)</th>
<th>Platelet Retention in VWD (%)</th>
<th>Ristocetin Aggregation in VWD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Phosphate (pH 7.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl Concentration (mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before dialysis</td>
<td>123</td>
<td>37</td>
<td>100</td>
</tr>
<tr>
<td>125</td>
<td>78</td>
<td>42</td>
<td>133</td>
</tr>
<tr>
<td>40</td>
<td>1.3</td>
<td>14</td>
<td>41</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>9</td>
<td>9.8</td>
</tr>
<tr>
<td>Control*</td>
<td>—</td>
<td>1</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* Saline substituted for factor VIII preparations.

with the normal factor VIII preparation. All factor VIII preparations were dialyzed against saline for 18 hr at 4°C, before testing the correcting activity on either platelet retention or ristocetin aggregation. In control experiments, dialysis fluid (saline) was added instead of the factor VIII preparations.

The influence of anti-slow- or anti-fast-moving component on the platelet retention of normal blood was tested as previously described for anti-factor VIII. 8 ml of normal blood was mixed with 50 μl of either anti-slow- or anti-fast-moving component. Platelet retention was evaluated according to Bowie. Control experiments were performed by adding 50 μl of normal rabbit serum.

The influence of the anti-slow- and anti-fast-moving component on the ristocetin aggregation of normal PRP was tested in a Payton Dual Channel aggregometer (Payton, Ontario, Canada). To 0.5 ml of PRP was added 5, 15, or 25 μl of antiserum. Ristocetin aggregation was performed as described above. The degree of aggregation was expressed as a percentage of the initial velocity of the control experiments in which an equal volume of normal rabbit serum was added.

RESULTS

Influence of Low-Ionic-Strength Dialysis on the Biologic Activities of Factor VIII

Dialysis of factor VIII at low ionic strength caused not only a decrease in factor VIII activity but also a reduction in correcting activity on the abnormal platelet retention and ristocetin aggregation of patients with VWD (Table I).

Immunologic Characterization of the Low-Ionic-Strength Components

The low-ionic-strength components showed partial identity with factor VIII when tested in immunodiffusion against antinormal factor VIII (Fig. 1A). When factor VIII dialyzed at low ionic strength was tested in immunodiffusion against antifactor VIII two precipitin lines were observed, each of which showed a reaction of identity with one of the two low-ionic-strength components (Fig. 1B). Application of the specific antisera against the low-ionic-strength components in immunodiffusion gave one line when tested against factor VIII dialyzed at low ionic strength and showed a reaction of identity with the corresponding component (Fig. 1C and D). The reaction of nonidentity between the slow- and fast-moving components indicated that these two components were antigenically different (Fig. 2).

Preparation of Antisera Against Low-Ionic-Strength Components

Antisera against the fast- and slow-moving components were prepared, and their specificity was established by cross-immunelectrophoresis with factor
Fig. 1. Agarose gel immunodiffusion. (A) Well No. 1 contained anti-factor VIII, No. 2 slow-moving component, No. 3 factor VIII, and No. 4 fast-moving component. (B) Well No. 1 contained anti-factor VIII, No. 2 slow-moving component, No. 3 factor VIII dialyzed at low ionic strength and No. 4 fast-moving component. (C) As (B), but anti-slow-moving component in No. 1. (D) As (B), but anti-fast-moving component in No. 1.
VIII dialyzed at low ionic strength (Fig. 3). Two peaks were obtained when antinormal factor VIII was applied to the agarose gel. Only one peak was observed, corresponding to the fast- and slow-moving components, when the antisera against the fast- and slow-moving components were applied to the gel. Combination of the antisera resulted in a pattern similar to the one obtained with antifactor VIII. The specificity of the two antisera was also demonstrated in immunodiffusion experiments, i.e., against slow- and fast-moving components and against factor VIII preparations which had been dialyzed against buffers of low ionic strength.

Properties of the Antisera Against the Low-Ionic-Strength Components

The anti-slow- and anti-fast-moving components both inhibited the platelet retention of normal blood (Table 2). A representative experiment on the reduction of the ristocetin aggregation of normal platelets by the antisera against the low-ionic-strength components is shown in Table 3. Comparable results were obtained in three other experiments. The factor VIII activity of normal plasma was only slightly inactivated by anti-slow-moving component (Fig. 4), although the latter's precipitin titer was comparable to that of the corresponding antibodies in anti-factor VIII (Fig. 3). Anti-fast-moving component was more effective in inactivating factor VIII, but the residual factor VIII activity was still higher than with anti-factor VIII (Fig. 4). The precipitin titer of anti-fast-moving component was about twice that of anti-factor VIII (Fig. 3). Combination of the two antisera did not increase the capacity to inhibit factor VIII. Both antisera removed factor VIII activity and factor VIII related antigen (FVIIIRA) from normal plasma, when insolubilized on Sepharose particles.7
Fig. 3. Cross-immunoelectrophoresis. The same factor VIII preparation dialyzed at low ionic strength was used in all four experiments. The respective agarose layers contained: (A) Anti-factor VIII (0.8%), (B) Anti-slow-moving component (1%), (C) Anti-fast-moving component (0.4%), (D) Anti-slow-moving component (1%) and anti-fast-moving component (0.4%).

Table 2. Effect of Antibodies Against Low-Ionic-Strength Components on the Platelet Retention (%) of Normal Blood

<table>
<thead>
<tr>
<th></th>
<th>Anti-slow-moving Component</th>
<th>Anti-fast-moving Component</th>
<th>Normal Rabbit Serum</th>
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</thead>
<tbody>
<tr>
<td>19</td>
<td>40</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>39</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>64</td>
<td></td>
</tr>
</tbody>
</table>

For method see ref. 2. Normal blood (8 ml) of three donors was mixed with antibody (50 μl) or normal rabbit serum (50 μl). Platelet retention test was performed according to Bowie.12

Table 3. Effect of Antibodies Against Low-Ionic-Strength Components on the Ristocetin Aggregation of Normal Platelets

<table>
<thead>
<tr>
<th>Amount of Antibody (μl)</th>
<th>Ristocetin Aggregation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-Factor VIII</td>
</tr>
<tr>
<td>5</td>
<td>63</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
</tr>
</tbody>
</table>

The degree of aggregation induced by ristocetin was expressed as the percentages of the initial velocity of the control experiments in which an equal amount of normal rabbit serum was added.
Factor VIII activity and FVIIIRA were completely abolished in at least 1 ml normal plasma by 2 mg insolubilized γ-globulin. After dissociation of the antigen–antibody complex, FVIIIRA was recovered.

Neutralization of Rabbit and Human Anti-Factor VIII by Low-Ionic-Strength Components

The factor VIII-neutralizing antibodies of rabbit anti-factor VIII were blocked by both components, as well as by equivalent amounts of normal factor VIII and factor VIII dialyzed at low ionic strength (Table 4). Equivalent amounts of normal factor VIII and factor VIII dialyzed at low ionic strength were obtained by dividing a factor VIII preparation into two parts, one of which was dialyzed against 0.01 M sodium phosphate (pH 7.0), and the other against 0.01 M sodium phosphate (pH 7.0) containing 125 mM NaCl. Both preparations were subsequently dialyzed against saline and then tested for their capacity to block the antibodies.

The factor VIII-neutralizing antibodies of human inhibitors were blocked by factor VIII, but not or only partially by equivalent amounts of factor VIII dialyzed at low ionic strength (Table 4). The low-ionic-strength components also caused only partial blocking of the neutralizing antibodies (Table 4).

Although the method applied is not very precise because the neutralizing capacity is determined by a complex assay with inherent variability, ten repeti-

Table 4. Anti-Factor VIII Neutralization by Low-Ionic-Strength Components of Factor VIII

<table>
<thead>
<tr>
<th>Antibody Neutralization Assay</th>
<th>% Factor VIII Activity Retained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slow-moving Component</td>
</tr>
<tr>
<td>Rabbit anti-VIII</td>
<td>74</td>
</tr>
<tr>
<td>Human anti-VIII</td>
<td>41</td>
</tr>
</tbody>
</table>

For details, see Methods. A constant volume of rabbit anti-factor VIII (25 µl) was mixed with a dilution of test material in saline (final volume 0.5 ml). Human anti-factor VIII 0.1 ml was mixed with a dilution of test material in saline (final volume 0.2 ml). Residual factor VIII activities were expressed as percentages of the activity in the control experiment where normal rabbit serum or normal human serum was added.
Fig. 5. Cross-immunoelectrophoresis of factor VIII after dialysis at low ionic strength and storage at 4°C, with antifactor VIII (0.8%). The peak with the intermediate mobility represents the third component.

Anomalies of the experiments shown in Table 4 with different antisera and factor VIII preparations gave consistent results: after dialysis against buffers of low ionic strength factor VIII never blocked the human inhibitors, whereas the rabbit antibodies were always blocked.

Third Component After Low-Ionic-Strength Dialysis

Recently a third component was detected after factor VIII preparations obtained by dialysis at low ionic strength had been stored at 4°C for several days. This component could only be demonstrated with anti-factor VIII (Fig. 5) and it did not precipitate with the antisera against the low-ionic-strength components. The third component was isolated by preparative polyacrylamide gel electrophoresis and was used for the immunization of rabbits. An antibody was obtained which, after absorption with the slow- and fast-moving components, proved to be specific for the third component. Preliminary experiments with this antibody (to be published) showed that the third component derived from the slow-moving component.

DISCUSSION

After dialysis against buffers of low ionic strength, factor VIII behaves as an aggregating series of homologous oligomers that are complexes of two different proteins. The immunologic studies reported here are an extension of those described before and reveal that these two proteins can be distinguished on the basis of specific antigenic structures. Preliminary experiments have indicated that reassociation does not occur at physiologic ionic strength. The fact that the factor VIII fraction isolated from normal plasma corrects the abnormal platelet
retention in a glass bead column as well as the abnormal ristocetin aggregation in VWD\textsuperscript{11,12} raises the question of whether the correcting activities can be attributed to one or both of these molecular species.

The finding that dialysis of factor VIII at low ionic strength decreases factor VIII procoagulant activity and reduces the correction of the abnormal platelet retention and ristocetin aggregation in VWD (Table 1) suggests that the intact aggregate is required for factor VIII activity and for the correcting activities. Recently, it has been demonstrated that infusion of factor VIII into dogs with VWD results in a correction of the bleeding time, indicating that factor VIII and von Willebrand factor are identical or are present in a complexed form,\textsuperscript{13} as had already been suggested.\textsuperscript{211} Because dialysis of factor VIII at low ionic strength results in a decrease in the correcting activities on platelet retention and ristocetin aggregation in VWD, it seems reasonable to assume that the intact aggregate is also required for the von Willebrand factor activity. In this respect the effect of dialysis of factor VIII at low ionic strength differs from the dissociation at high salt concentration which yields a HMW subunit with von Willebrand factor activity and a LMW subunit with factor VIII procoagulant activity. The two types of subunits also differ in their immunologic properties. Whereas the LMW subunit retains factor VIII antigenic determinants, undetectable by immunoprecipitation or radioimmunoassay, the factor VIII antigenic determinants on the HMW subunit are identifiable by methods based on immunoprecipitation, radioimmunoassay and antibody neutralization assay. However, immunoprecipitation shows that the low-ionic-strength components have different antigenic determinants (Fig. 2).

The factor VIII neutralizing antibodies of human inhibitors were blocked by factor VIII but not or only partly by equivalent amounts of factor VIII dialyzed at low ionic strength (Table 4). The low-ionic-strength components also blocked the neutralizing antibodies only partly. Dialysis of factor VIII at low ionic strength was shown to lead to a decrease of factor VIII activity (Table 1). The decreased factor VIII activity found in the experiments was accompanied by the reduction of an antigenic determinant(s) closely related to factor VIII function. The intact aggregate, therefore, was necessary not only for factor VIII procoagulant activity but probably also for the antigenic determinant(s) detected by human inhibitors.

The finding that the factor VIII-neutralizing antibodies of rabbit antifactor VIII were blocked by the components and by equivalent amounts of both normal factor VIII and factor VIII dialyzed at low ionic strength (Table 4) indicated at least a partial difference between the antigenic determinants required for blocking of the factor VIII-neutralizing antibodies in human and rabbit antisera. Antibodies specific for the low-ionic-strength components (Fig. 3) inhibited factor VIII activity in normal plasma, but the residual factor VIII activity was higher than that after inhibition with antibodies directed against intact factor VIII (Fig. 4). This finding might also indicate that the components lacked antigenic determinants closely related to the factor VIII activity site. Insolubilized antibodies against both types of components removed factor VIII activity and FVIIIIRA almost completely from normal plasma,\textsuperscript{1} suggesting that the residual factor VIII activity after incubation with the antisera against the
low-ionic-strength components in fluid phase was based on the presence of non-inhibitory antibodies. Noninhibitory antibodies could protect factor VIII from inactivation by inhibitory antibodies forming a steric hindrance or inducing a conformational change. Similar effects have been described for antibodies against enzymes.14,15

The antisera against both low-ionic-strength components reduced the platelet retention of normal blood and the ristocetin aggregation of normal platelets (Tables 2 and 3) and suggested that both kinds of antibodies interfered with the von Willebrand factor activity.

The HMW subunit has properties in common with the low-ionic-strength components: it has no factor VIII activity, it precipitates with rabbit antifactor VIII sera, and it neutralizes rabbit antibodies to factor VIII. These findings suggest that the HMW subunit is composed at least partially of the low-ionic-strength components. Unlike the HMW subunit, the low-ionic-strength components have no von Willebrand factor activity and only neutralize human factor VIII inhibitors partially, if at all. The antisera against the low ionic strength components do, however, interfere with von Willebrand factor activity.

Thus far the discussion has concerned the effect of dialysis of factor VIII against buffers of low ionic strength on the inactivation of both factor VIII and the von Willebrand factor and the effect of dialysis on antigenic determinants closely related to factor VIII function. With respect to the mode of interaction of these components, it has been suggested1 that these components are non-covalently linked in the native factor VIII molecule. On the other hand, it has recently been shown that factor VIII is exceedingly sensitive to rapid fragmentation by low concentrations of various proteolytic enzymes.16-18 Therefore, the possibility that the two components are the result of the breakdown of factor VIII due to contamination with proteases must also be considered. There are, however, several factors which do not support this mechanism.

(1) The pronounced effect of the ionic strength on the native structure of factor VIII.1 Minor changes in the ionic strength of the medium usually have little or no significant effect on proteolytic activity.

(2) The effect of low-ionic-strength dialysis on the structure of factor VIII appears to be ion specific. The formation of low-ionic strength components is not observed when barbital-HCl, collidine-HCl, or imidazol-HCl is substituted for sodium phosphate buffer.19 Triethanol amine-HCl is as effective as phosphate but barbital-HCl has only a slight effect at the same ionic strength and pH.19 In general, proteolysis does not show the same degree of ion specificity.

These findings suggest that the cleavage of factor VIII at low ionic strength and neutral pH is nonproteolytic in nature. On the other hand, studies with proteolytic inhibitors have been inconclusive. Soya bean trypsin inhibitor (1 mg/ml) and e-amino caproic acid (5 x 10^{-2}M) have virtually no effect on the formation of the low-ionic-strength components. However, this process is prevented by diisopropylfluorophosphate (1 mg/ml), whereas phenylmethylsulfonylfluoride (1 mg/ml) has only a slight effect. It is clear, however, that if factor VIII is affected proteolytically this process must be rather specific. This conclusion can be inferred from the striking linearity of the curve of log mobil-
ity of the intermediate fragments (having approximately constant charge-to-mass ratios') during gel electrophoresis, plotted against log band number. The observation that the final components have relatively high molecular weights and are stable at low ionic strength is consistent with cleavage as a result of limited proteolysis. Whether cleavage of the native factor VIII structure is due to proteolytic breakdown or to the disruption of noncovalent bonds, it is evident that the fragmentation of factor VIII at low ionic strength can contribute to the further characterization of the factor VIII–von Willebrand factor complex.

ACKNOWLEDGMENT

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Immunologic studies on human factor VIII (anti-hemophilic factor A, AHF) components produced by low-ionic-strength dialysis

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