

## SUBCELLULAR PLATELET FACTOR VIII ANTIGEN AND VON WILLEBRAND FACTOR\*

By RALPH L. NACHMAN AND ERIC A. JAFFE‡

(From the Division of Hematology, Department of Medicine, Cornell University Medical College,  
New York 10021)

Factor VIII is a plasma protein which in the purified state has a mol wt in the range of  $1.1 \times 10^6$  (1). Three distinct properties have been associated with this plasma protein: (a) factor VIII clot-promoting function ( $VIII_{AHF}$ ),<sup>1</sup> an activity which corrects the coagulation abnormality of plasma from a patient with classic hemophilia (hemophilia A); (b) factor VIII antigen ( $VIII_{AGN}$ ), an antigen identified in precipitin assays by heterologous antibodies, normal or increased in hemophilic plasma but decreased in plasma of patients with von Willebrand's disease; and (c) von Willebrand factor ( $VIII_{VWF}$ ), an activity deficient in von Willebrand's disease which has been identified by an abnormality of the bleeding time or by in vitro assays of platelet function (ristocetin-induced aggregation and retention in glass bead columns). Factor VIII refers to a protein or protein system in normal plasma which is responsible for these three activities. We have previously demonstrated that cultured human endothelial cells synthesize and release a protein(s) which has  $VIII_{AGN}$  and  $VIII_{VWF}$  activity (2, 3). Endothelial cells thus synthesize and release activities which significantly influence platelet function. Recent studies (4, 5) suggest that the factor VIII system may be closely associated with the platelet surface.

The following studies were undertaken to localize and characterize the separate components of the factor VIII system in isolated human platelets. This paper demonstrates the presence of  $VIII_{AGN}$  and  $VIII_{VWF}$  in subcellular platelet membrane and granule fractions.

### Materials and Methods

*Platelet Subcellular Fractions.* Human platelets were processed as previously described (6). Fresh platelets (processed the same day as drawn) as well as outdated platelet packs (kindly provided by the New York Blood Center) were utilized for these studies. Platelet membrane and granule fractions were prepared by sucrose density gradient ultracentrifugation of platelet homogenates (7). The membranes and granules were washed twice in 30 volumes of buffered NaCl solution (pH 7.4, 0.15 M NaCl-0.0175 M phosphate) containing the broad spectrum protease inhibitor toluene sulfonyl fluoride (0.4 mM) and the cathepsin inhibitor, N-carbobenzoxy- $\alpha$ -L-glutamyl-L-tyrosine (0.5 mM) (8). In some experiments, the platelets were processed from the platelet-rich plasma step to the final subcellular fractions in the presence of the protease and cathepsin inhibitors. The subcellular fractions pooled from multiple units of platelets were stored frozen at  $-20^{\circ}\text{C}$ . The platelet-soluble layer was cleared of residual subcellular material by ultracentrifugation at 100,000  $g$  in a Beckman L2-65 ultracentrifuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.), dialyzed versus Ardlie buffer salt solution (8), and stored frozen at  $-20^{\circ}\text{C}$ . The buffer was modified from the original

\*This investigation was supported by NIH grant HL-14810 and a grant from the Arnold R. Krakower Hematology Foundation.

‡ Career Scientist of the Health Research Council of the City of New York (Contract I-808).

*Abbreviations used in this paper:*  $VIII_{AGN}$ , factor VIII antigen;  $VIII_{AHF}$ , factor VIII procoagulant activity;  $VIII_{VWF}$ , von Willebrand factor; SDS, sodium dodecyl sulfate.

by omitting glucose, heparin, albumin, and apyrase. For solubilization the subcellular fractions were incubated for 18 h at 4°C in Ardlie buffer containing 0.1% Lubrol PX (Sigma Chemical Co., St. Louis, Mo.). The soluble protein solutions were separated from the residual insoluble material by ultracentrifugation at 100,000 *g* for 1 h. The excess Lubrol PX was removed from the supernates containing the solubilized subcellular proteins by the method of Beckman et al. (9). The soluble subcellular supernates were stirred for 30 min at 4°C with an equal volume of Amberlite resin (XAD-2, Mallinckrodt Chemical Works, St. Louis, Mo.) suspended in 0.01 M Tris HCl buffer, pH 7.5, in order to absorb the Lubrol. The resin was then removed by centrifugation at 1,000 *g* for 10 min. The protein recovery in the Lubrol PX-solubilized subcellular fractions in multiple experiments varied between 50 and 60% of the total particulate protein.

**Antisera.** Monospecific rabbit antihuman factor VIII was obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. This antibody precipitates VIII<sub>AGN</sub>, inhibits VIII<sub>AHF</sub> of normal plasma, and inhibits ristocetin-induced platelet aggregation (VIII<sub>VWF</sub>). On immunodiffusion analysis, this antibody reacted with a single line of identity when tested against normal plasma, hemophilic plasma, and human factor VIII concentrate (Hemofil®, Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.). The antibody did not form a precipitin line with plasma from patients with severe von Willebrand's disease. The antibody also formed a line of identity with two other antisera monospecific for factor VIII (2, 3) when all three were reacted against factor VIII concentrate (Hemofil®).

**γ-Globulin Fractions.** γ-Globulin fractions of the antiserum and normal rabbit γ-globulin were prepared as described by Kolb et al. (10). The antiserum was dialyzed for 24 h at 4°C against 500 volumes of sodium phosphate buffer (0.01 M phosphate, pH 7.0). After removal of insoluble protein by centrifugation at 3,000 *g* for 30 min, the dialyzed sample was applied to a 1.5 × 28.5 cm TEAE-cellulose column equilibrated with the dialysis buffer and the breakthrough protein collected and concentrated by precipitation at 0°C with ammonium sulfate at 50% saturation. The precipitate was washed three times with 50% saturated ammonium sulfate, dissolved, and dialyzed against 0.15 M NaCl. Insoluble γ-globulin preparations were prepared by coupling the isolated γ-globulin to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) (11).

**Semipurified Plasma Factor VIII.** Semipurified plasma factor VIII was prepared from a commercially available factor VIII concentrate (Hemophil®). 20 ml of Hemophil® containing 500 U of VIII<sub>AHF</sub> was chromatographed on a 5 × 80 cm Sepharose 4B column in phosphate-buffered saline (0.145 M NaCl, 0.01 M phosphate, pH 7.4) at room temperature at an elution rate of 80 ml/h. The void volume was determined with Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.). Void volume fractions were concentrated approximately 20-fold using a hollow fiber ultrafilter device (Bio Fiber 80-Minitube, Bio-Rad Laboratories, Richmond, Calif.). The final preparation which contained 0.32 mg/ml protein had 32 U of VIII<sub>AGN</sub>/ml as determined by radioimmunoassay (kindly performed by Dr. Leon Hoyer, University of Connecticut) (12). On sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis after reduction with dithiothreitol, 60–70% of the protein in this preparation as determined by densitometric scan moved as a single band in the mol wt range of 230,000.

**Quantitative Immunoelectrophoresis of VIII<sub>AGN</sub> in Subcellular Fractions.** This was carried out by a modification of the Laurell quantitative immunoelectrophoretic technique as described by Zimmerman et al. (13). The antiserum was diluted in the agarose 1:50. 10 μl of the solution containing the antigen were deposited in 3-mm diameter wells. A standard curve was constructed by testing whole plasma in serial dilutions. 1 U of the antigen is the amount present in 1 ml of pooled normal plasma prepared from 10 normal male adult subjects.

**Immunoabsorption of Subcellular VIII<sub>AGN</sub>.** 1 ml of solubilized membrane protein solution (varying in different experiments from 2 to 2.25 mg/ml) was incubated at room temperature with 0.5 ml insolubilized anti-VIII Sepharose (3.5 mg globulin). The mixture was shaken at room temperature on a rotating tumbler (Labindustries, Berkeley, Calif.) for 2 h. The clear membrane protein supernate was removed after centrifugation at 8,000 *g* for 20 min. Identical insoluble antibody immunoabsorption studies were performed with the soluble granule protein solution (1–2.2 mg/ml). Control immunoabsorption studies of the subcellular fractions were performed using insoluble normal rabbit γ-globulin.

**SDS Polyacrylamide Gel Electrophoresis.** SDS polyacrylamide gel electrophoresis (5% gels) was performed as described by Weber and Osborn (14). Samples for analysis were added to an equal volume of a solution containing 10 M urea, 2% SDS, and 14 mM dithiothreitol and boiled for 5 min.

Precipitates for gel electrophoresis were solubilized in 100  $\mu$ l of 10 M urea-2% SDS-14 mM dithiothreitol and boiled before application on the gel. The gels were stained with Coomassie brilliant blue. Densitometric scans of gels were carried out in a Gilford Model 240 spectrophotometer equipped with a gel scanning attachment (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and a Densicord recorder equipped with an integrator (Photovolt Corp., New York).

The molecular weight of VIII<sub>AGN</sub> was determined by SDS acrylamide gel electrophoresis as described (14). Molecular weight markers included phosphorylase A, mol wt 94,000 (Sigma Chemical Co.); ovalbumin, mol wt 43,000 (Pharmacia Fine Chemicals Inc.); chymotrypsin, mol wt 25,000 (Calbiochem, San Diego, Calif.); and  $\alpha$ -2-macroglobulin subunit, mol wt 185,000 (Kindly provided by Dr. Peter Harpel).

**Subcellular Platelet VIII<sub>VWF</sub> Activity.** This was assayed by the ristocetin method as previously described (3, 15, 16). Washed normal human platelets were prepared using the Ardlie buffer salt system (8). For these purposes the buffer was modified by omitting calcium and adding 1 mM adenosine to all wash solutions and increasing the number of washes to five. The assay was performed in an aggregometer (Payton Dual Channel, Payton Assoc., Buffalo, N. Y.) using 350  $\mu$ l of washed platelets, 50  $\mu$ l of the material to be tested, and ristocetin (Abbott Laboratories, Chemical Marketing Div., Chicago, Ill.) at a final concentration of 1.5 mg/ml.

**Ristocetin-Platelet Membrane Protein Interaction.** This was studied by comparing the SDS gel electrophoretic patterns of the solubilized membrane polypeptides after incubation with varying amounts of ristocetin. The membrane protein solutions were incubated with the ristocetin for 1 h at 37°C and the precipitate pelleted at 8,000 *g* for 20 min. The supernates were incubated with equal volumes of the urea-SDS-dithiothreitol solution, boiled, and analyzed by polyacrylamide gel electrophoresis. Similarly the precipitates were solubilized in 100  $\mu$ l of the same solution, boiled, and analyzed on disc gels.

**Subcellular Platelet VIII<sub>AHF</sub> Activity.** This was assayed by a one-stage method using congenital VIII<sub>AHF</sub>-deficient human plasma as substrate (17). Pooled normal human plasma was used as the standard and VIII<sub>AHF</sub> values expressed as units per milliliter. 1 U of VIII<sub>AHF</sub> is defined as that activity present in 1 ml of pooled platelet-poor plasma from 10 or more normal donors.

## Results

**Quantitation of Factor VIII<sub>AGN</sub> in Platelet Subcellular Fractions.** The antigen was detected in the soluble protein obtained from both the subcellular granule and membrane fractions (Table I). No antigenic activity was identified in the cytosol-soluble fraction. The VIII<sub>AGN</sub> in the subcellular fractions reacted identically to the plasma VIII<sub>AGN</sub> standard in quantitative immunoelectrophoresis. When the heights of the rocket precipitins obtained from serial dilutions of the membrane and granule VIII<sub>AGN</sub> and the plasma standard were graphically plotted, the three lines connecting the three different sets of points were parallel.

TABLE I  
*Quantitation of VIII<sub>AGN</sub> in Platelet Subcellular Fractions*

Fraction	Protein	VIII <sub>AGN</sub> * U/mg	VIII <sub>AGN</sub> † U/ml blood
	mg/ml		
Granules	1.13	1.54	0.113
Membranes	2.36	3.39	0.048
Soluble	2.12	0	—

\* 1 U of VIII<sub>AGN</sub> is defined as the amount of VIII<sub>AGN</sub> present in 1 ml pooled platelet-poor plasma from 10 normal donors.

† Calculated from the protein distribution of platelet fractions obtained from 1 unit (450 ml) of blood. Granules 22%, and membranes 4.3% (7). 1 U of platelets contains 150 mg protein (18, 19).

The VIII<sub>AGN</sub> measured was the same using fresh as well as outdated platelets. Both subcellular fractions were considerably enriched in factor VIII<sub>AGN</sub> when compared to plasma. The plasma standard contained 1 U VIII<sub>AGN</sub>/70 mg protein (1 ml platelet-poor plasma) or 0.014 U/mg. Thus the platelet granules were enriched over 100-fold and the membranes were enriched over 200-fold in VIII<sub>AGN</sub> compared to plasma. From previously published data it was possible to calculate the VIII<sub>AGN</sub> content of whole blood attributable to the platelet fractions. Isolated platelet granules and membranes account for 22 and 4.3% respectively, of the platelet protein separated from 1 unit (450 ml) of blood (7) and 1 unit of platelets is equivalent to 150 mg of protein (18, 19). Thus 1 ml of whole blood contains 73  $\mu$ g of granule protein. This amount of granule protein contains 0.113 U of VIII<sub>AGN</sub> (Table I). Similarly, 1 ml of whole blood contains 14  $\mu$ g of membrane protein ( $[150 \times 0.043]/450$ ). This amount of membrane protein contains 0.048 U of VIII<sub>AGN</sub> (Table I). Thus the platelets in 1 ml of whole blood contain approximately 15% of the amount of VIII<sub>AGN</sub> present in 1 ml of platelet-poor plasma.

*Identification of Factor VIII<sub>AGN</sub> Subunit in Platelet Subcellular Fractions.* For these studies, the subcellular fractions (isolated membranes or granules) were solubilized in Lubrol and then exposed to Amberlite to remove the excess detergent. The residual soluble proteins were incubated with insoluble rabbit antibody to human factor VIII and the immune complex removed by centrifugation. The absorbed and unabsorbed protein solutions were analyzed by SDS acrylamide gel electrophoresis for the presence or absence of a protein which moved with the same electrophoretic mobility as partially purified plasma factor VIII<sub>AGN</sub>.

**IMMUNOABSORPTION OF THE SOLUBLE PLATELET MEMBRANE PROTEINS.** The soluble platelet membrane preparation contained numerous polypeptide bands (Fig. 1), one of which moved with the same electrophoretic mobility as partially purified plasma factor VIII<sub>AGN</sub> (approximate mol wt 230,000). This latter band was the only one removed from the membrane protein solution by absorption with insoluble anti-VIII. There was no effect on the membrane protein solution after exposure to insoluble normal rabbit  $\gamma$ -globulin.

**IMMUNOABSORPTION OF THE SOLUBLE PLATELET GRANULE PROTEINS.** The soluble platelet granule preparation contained numerous polypeptide bands (Fig. 2), one of which moved with the same electrophoretic mobility as partially purified plasma factor VIII<sub>AGN</sub>. This latter band was the only one removed from the granule protein solution by absorption with insoluble anti-VIII. There was no effect on the membrane protein solution after exposure to insoluble normal rabbit  $\gamma$ -globulin.

**CYTOSOL PROTEINS.** The soluble cytosol platelet protein preparation contained numerous polypeptides (Fig. 3); however, there was no band in the factor VIII 230,000 mol wt region.

*Subcellular Platelet VIII<sub>VWF</sub>.* VIII<sub>VWF</sub> was assayed by determining the presence of an activity in the various subcellular protein solutions which supported ristocetin-induced aggregation of washed normal human platelets. Soluble subcellular granule and membrane protein supported ristocetin-induced aggregation while the soluble cytosol fraction did not (Fig. 4). Washed platelets in buffer did not aggregate after the addition of ristocetin alone (not shown). No attempts were made to quantitate the VIII<sub>VWF</sub> activity in the separate fractions.

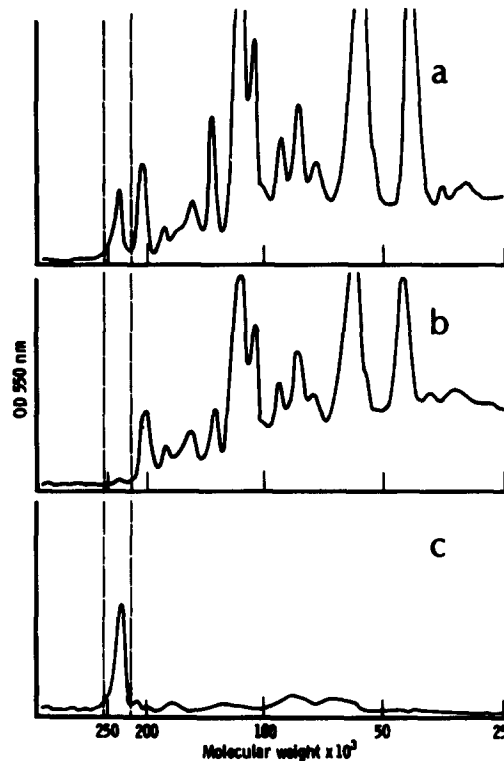


FIG. 1. Immunoblotting of platelet membrane factor VIII<sub>AGN</sub>. Densitometric scans of SDS acrylamide gels of: (a) membrane soluble protein solution—50  $\mu$ l (2.2 mg/ml) was added to 50  $\mu$ l of a solution containing 10 M urea, 2% SDS, and 14 mM dithiothreitol and boiled for 5 min. 25  $\mu$ l bromphenol blue dye marker was added and 50  $\mu$ l (44  $\mu$ g) applied to the gel. (b) Identical experiment as described in a except that the membrane protein solution was first absorbed with insoluble anti-VIII. (c) Semipurified plasma factor VIII (4  $\mu$ g). Coomassie blue stained gels with anode to the right. The vertical dashes enclose the factor VIII<sub>AGN</sub> subunit region.

*Platelet Membrane Protein Ristocetin Interaction.* An attempt was made to monitor the ristocetin-platelet membrane protein reaction by comparing the SDS gel electrophoretic patterns of the membrane polypeptides after exposure to variable amounts of ristocetin (Fig. 5). Ristocetin precipitated membrane proteins including VIII<sub>AGN</sub> with the maximum effect occurring at a ristocetin concentration of 1 mg/ml. This appeared to be a nonspecific reaction as different concentrations of ristocetin precipitated the same proportions of both factor VIII<sub>AGN</sub> and an unrelated membrane protein labeled "X" (Fig. 6).

*Subcellular Platelet Factor VIII<sub>AHF</sub>.* None of the subcellular fractions contained VIII<sub>AHF</sub> as assayed in an VIII<sub>AHF</sub>-deficient human plasma system (Table II). The inhibitors used in processing the platelet subcellular fractions (N-carbobenzoxy- $\alpha$ -L-glutamyl-L-tyrosine and toluene sulfonyl fluoride) had no effect on VIII<sub>AHF</sub> as assayed in the human plasma standard.

### Discussion

Subcellular fractions derived from normal human platelets have been shown in these studies to contain significant amounts of VIII<sub>AGN</sub> and VIII<sub>VWF</sub> activity. The

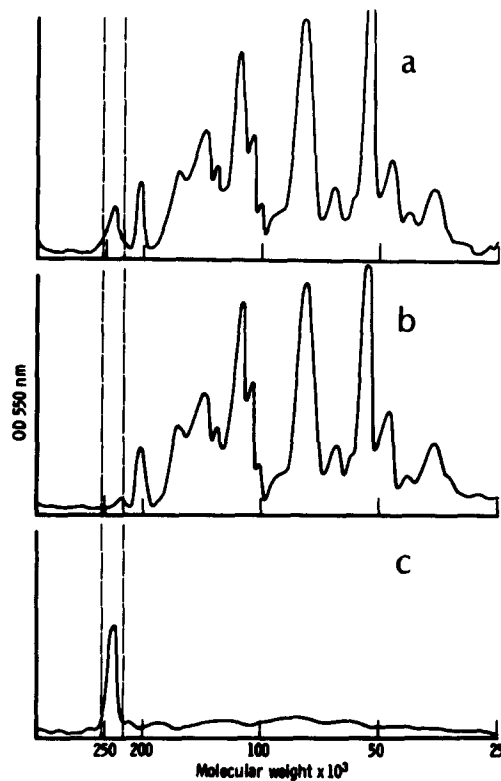


FIG. 2. Immunoabsorption of platelet granule factor VIII<sub>AGN</sub>. Densitometric scans of SDS acrylamide gels of: (a) granule soluble protein solution—50  $\mu$ l (1.9 mg/ml) was added to 50  $\mu$ l of a solution containing 10 M urea, 2% SDS, and 14 mM dithiothreitol and boiled for 5 min. 25  $\mu$ l bromphenol blue dye marker was added and 50  $\mu$ l (38  $\mu$ g) of the solution applied to the gel. (b) Identical experiment as described in a except that the membrane protein solution was first absorbed with insoluble anti-VIII. (c) Semipurified plasma factor VIII (4  $\mu$ g). Coomassie blue stained gels with anode to the right. The vertical dashes enclose the factor VIII<sub>AGN</sub> subunit region.

VIII<sub>AGN</sub> was identified in the subcellular fractions by quantitative immunoelectrophoresis and immunoabsorption using insoluble antibodies. The specificity of these techniques is dependent on the specificity of the antiserum used. The rabbit anti-VIII employed in these studies had the same inhibitory properties as a highly specific rabbit anti-VIII which we used and characterized previously (2). On immunodiffusion analysis, the rabbit anti-VIII reacted with a single line of identity when tested against human plasma, hemophilic plasma, and a concentrate of human plasma factor VIII but did not form a precipitin line with plasma from patients with severe von Willebrand's disease. The antibody also formed a line of identity with two other antisera monospecific for factor VIII (2, 3) when all three were reacted against factor VIII concentrate. This lack of a precipitin line against von Willebrand's plasma strongly suggests that the anti-VIII sera did not contain contaminant antibody against cold-insoluble globulin since patients with von Willebrand's disease have normal plasma levels of cold-insoluble globulin (20). This negative finding is important because on SDS acrylamide gel electro-

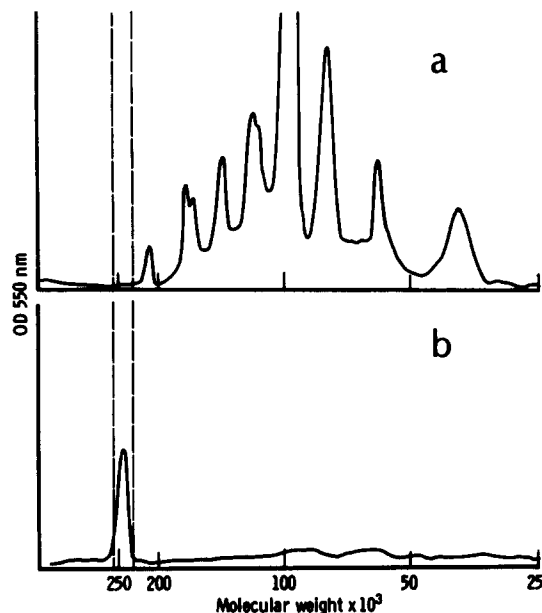


FIG. 3. Polypeptides of platelet cytosol-soluble subcellular fraction. Densitometric scans of SDS acrylamide gels of: (a) cytosol protein solution — 50  $\mu$ l (2 mg/ml) was added to 50  $\mu$ l of a solution containing 10 M urea, 2% SDS, and 14 mM dithiothreitol, and boiled for 5 min. 25  $\mu$ l bromphenol blue dye marker was added and 50  $\mu$ l (40  $\mu$ g) of the solution added to the gel. (b) Semipurified plasma factor VIII (4  $\mu$ g). Coomassie blue stained gels with anode to the right. The vertical dashes enclose the factor VIII<sub>AGN</sub> subunit region.

phoresis after reduction, cold-insoluble globulin also has a mol wt of approximately 230,000.

Analysis of the membrane and granule-soluble protein fractions by SDS acrylamide gel electrophoresis demonstrated a polypeptide in both fractions with the same molecular weight as human plasma factor VIII. Insolubilized rabbit anti-VIII removed only this peptide from the membrane and granule protein preparations. In other work we have shown that the insoluble anti-VIII removed only a band of the same molecular weight from a preparation of concentrated human plasma factor VIII.<sup>2</sup> These observations strongly suggest that the molecular species in the platelet fractions is identical to human plasma VIII<sub>AGN</sub>. VIII<sub>VWF</sub> was demonstrated in the platelet subcellular fractions by the presence of an activity in the solubilized protein solutions which supported ristocetin-induced aggregation of washed platelets. No VIII<sub>AHF</sub> was detected in these preparations.

Recent studies by others have also suggested that plasma VIII<sub>AGN</sub> and VIII<sub>VWF</sub> activity is available on the platelet surface (4, 5). Our studies demonstrate that the intracellular platelet granule population contains a significant reservoir of platelet VIII<sub>AGN</sub> and VIII<sub>VWF</sub>. It is possible that granule VIII<sub>AGN</sub> and VIII<sub>VWF</sub> serves as a storage source of surface membrane VIII<sub>AGN</sub> and VIII<sub>VWF</sub>. Weiss et al. (21) have shown that factor VIII, which is necessary for ristocetin-induced

<sup>2</sup>Jaffe, E.A., and R. L. Nachman. Manuscript in preparation.

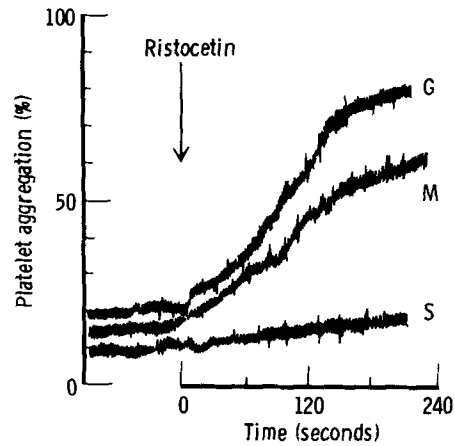


FIG. 4. Ristocetin-induced aggregation of washed normal platelets. G, granule protein solution; M, membrane protein solution; and S, soluble layer from platelet subcellular fractionation. Protein was 2 mg/ml in the three solutions. 350  $\mu$ l of washed platelets (300,000/ $\mu$ l) and 50  $\mu$ l of the test protein solution were mixed in the cuvette for approximately 60 s and then ristocetin was added at zero time as 20  $\mu$ l of a 30 mg/ml solution (final concentration 1.5 mg/ml).

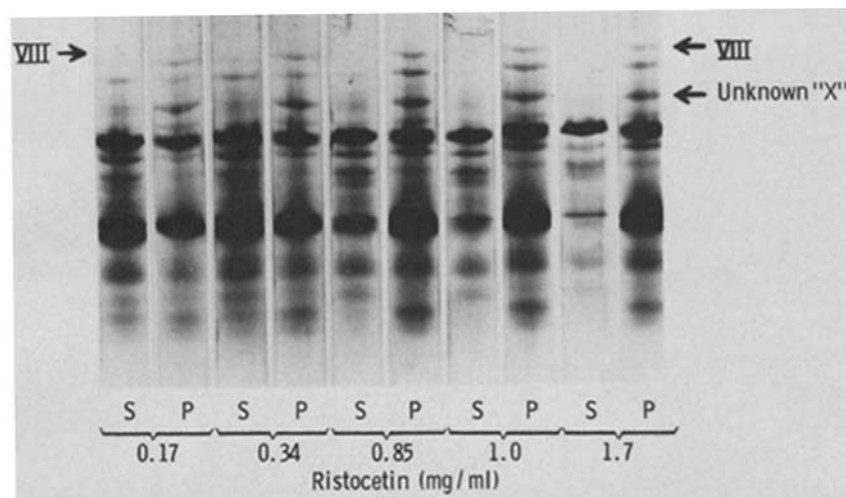


FIG. 5. SDS acrylamide gel electrophoresis of membrane protein solutions (S, supernate and P, precipitate) after exposure to variable amounts of ristocetin as indicated for 60 min at 37°C. The precipitates were pelleted and processed as described in Fig. 1. 50  $\mu$ l were applied to the gels. 45  $\mu$ l of the residual supernates were mixed with 50  $\mu$ l of the urea SDS dithiothreitol solution and processed as described in Fig. 1. 50  $\mu$ l of solution were applied to the gel. VIII indicates the position of VIII<sub>AGN</sub>. X is an unidentified membrane band which was compared to VIII<sub>AGN</sub> in Fig. 6.

aggregation, is removed from the platelets by repeated washing but that some remains within the platelet and is subsequently made available in a washed buffer system (16). Similarly, Howard et al. (4) have shown that lysed platelets contain greater amounts of VIII<sub>AGN</sub> than the corresponding suspension of whole



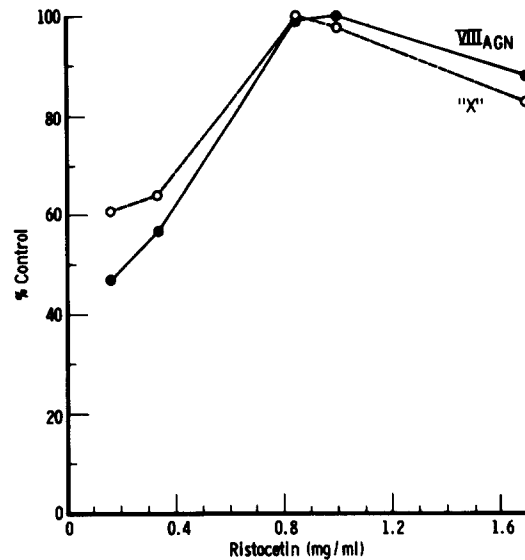


FIG. 6. Densitometric scan of VIII<sub>AGN</sub> compared to an unidentified membrane band "X" in the ristocetin membrane protein precipitates shown in Fig. 5. The density of the respective bands was compared to the amounts present in an equivalent concentration of membrane protein solution unexposed to the ristocetin.

TABLE II  
VIII<sub>AHF</sub> in Platelet Subcellular Fractions

Fraction	Protein <i>mg/ml</i>	VIII <sub>AHF</sub> * <i>U/ml</i>
Granules	1.13	<0.01
Membranes	2.36	<0.01
Soluble	2.12	<0.01

\* 1 U of VIII<sub>AHF</sub> is defined as the amount of VIII<sub>AHF</sub> present in 1 ml pooled platelet-poor plasma from 10 normal donors.

platelets. It is not clear from our studies whether the intracellular platelet VIII<sub>AGN</sub> and VIII<sub>VWF</sub> are present in a specific granule compartment.

The total amount of platelet VIII<sub>AGN</sub> is surprisingly large in terms of that amount present in normal platelet-poor plasma. Thus the platelets in 1 ml of blood contain approximately 15% of the VIII<sub>AGN</sub> present in 1 ml of platelet-poor plasma (Table I). This should be compared to platelet fibrinogen which also occupies a plasma membrane and intracellular granule compartment (22). Platelet fibrinogen represents approximately 10% of the total platelet protein (19). Thus 1 ml of blood contains approximately 33  $\mu$ g of platelet fibrinogen which represents roughly 1% of the fibrinogen in 1 ml of platelet-poor plasma. The large relative amounts of VIII<sub>AGN</sub> (and presumably equally large amounts of VIII<sub>VWF</sub>) intimately associated with human platelets further emphasizes the importance of this protein(s) in normal hemostasis.

The relationship of the factor VIII system to normal platelet physiology has

been partially clarified in recent years by the study of patients with von Willebrand's disease. This genetically transmitted bleeding disorder is characterized by a decreased level of plasma factor VIII and a prolonged bleeding time (23). The prolonged bleeding time in most patients is closely correlated with a decreased level of factor VIII<sub>VWF</sub> as measured by the ristocetin assay (16). Our recent studies which demonstrate that endothelial cells synthesize and release VIII<sub>AGN</sub> (2) and release VIII<sub>VWF</sub> (3), a protein which significantly influences normal platelet function, further substantiate the importance of platelet-endothelial cell interactions in normal hemostasis (24). The present studies clearly demonstrate that VIII<sub>VWF</sub> activity is present on the platelet membrane. A potential physiologic implication of our observations is that circulating platelets normally adsorb endothelial cell-synthesized VIII<sub>VWF</sub> which in some unknown manner enables the platelet to interact with damaged vessel surfaces. Our contention is supported by a recent immunofluorescence study showing that endothelial cells in patients with severe von Willebrand's disease do not contain VIII<sub>AGN</sub> (25). The precise *in vivo* physiologic counterpart of defective ristocetin aggregability and diminished platelet adhesiveness to glass bead columns is not known. However, it has been recently demonstrated that platelets in the blood of patients with von Willebrand's disease adhere poorly to the subendothelial surface of rabbit aorta (26). The role of the intracellular granule storage site of VIII<sub>VWF</sub> in this hemostatic process remains to be clarified. The only other cell system in the body, besides endothelial cells, which stains for VIII<sub>AGN</sub> with highly specific antisera are megakaryocytes and platelets (27). Thus at least theoretically these cells might also synthesize VIII<sub>AGN</sub>. Platelets, however, have no DNA and only a vestigial protein synthetic apparatus (18). Whether megakaryocytes synthesize VIII<sub>AGN</sub> remains to be determined.

One of the more intriguing and puzzling features of factor VIII physiology is the role of ristocetin in inducing platelet aggregation. Our studies demonstrated that ristocetin precipitated membrane VIII<sub>AGN</sub> in a relatively nonspecific reaction. It is not clear how this precipitation reaction involving soluble proteins relates to the induction of the aggregation response in the presence of whole cells and surface VIII<sub>VWF</sub>. One possibility is interaction and possible modification of VIII<sub>VWF</sub> carbohydrate side chains. Studies on bovine plasma factor VIII have indicated that the integrity of carbohydrate side chains on the VIII molecule(s) are necessary for subsequent aggregation responses (28, 29). Thus bovine plasma factor VIII preincubated with galactose oxidase no longer induced platelet aggregation. However, human plasma factor VIII preincubated with neuraminidase supported platelet aggregation in the absence of added ristocetin (30). These observations raise the possibility that ristocetin alters platelet surface VIII<sub>VWF</sub> so as to sterically reorient unavailable sites or mask or alter carbohydrate side chains, thus allowing the protein to support cell to cell interactions.

Finally, our studies again raise some unanswered questions regarding the macromolecular complex structure of the factor VIII system. Why don't platelets contain subcellular VIII<sub>AHF</sub>? One possibility which has to be considered is that the procoagulant site (the AHF activity) was destroyed or damaged in the process of the preparation of the subcellular fractions. We have previously demonstrated that platelets contain potent intracellular proteases such as cathepsin A which

might destroy the procoagulant activity (31). However, when platelet subcellular fractions were processed in buffer containing inhibitors of both cathepsin and proteolytic enzymes, VIII<sub>AHF</sub> was still undetectable. The use of fresh platelets (processed the same day as drawn) as well as outdated platelet packs had no effect on the presence or absence of VIII<sub>AHF</sub> activity in the platelet fractions. It is thus unlikely that proteolytic degradation occurred due to protracted in vitro storage. The absence of the procoagulant activity might also be due to dissociation or masking of a critical site due to proteolytic or other effects of platelets on the VIII system both in vivo and in vitro before fractionation and the addition of the protease inhibitors. Another possibility which is raised by our previous studies (2, 3) is that the factor VIII system is a macromolecular complex with VIII<sub>AGN</sub> and VIII<sub>VWF</sub> activity residing on one molecular species and VIII<sub>AHF</sub> residing on another molecular species.

We suggest as a working hypothesis that endothelial cells synthesize and release and platelets adsorb a carrier or inactive precursor molecule which because it possesses both VIII<sub>AGN</sub> and VIII<sub>VWF</sub> is able to support normal platelet function. This precursor as it circulates in the plasma is converted to that configuration necessary for VIII<sub>AHF</sub> activity after interaction at a remote site either through contact with a second cell type or by interaction with one or more plasma enzymes. The clarification of these relationships awaits further study.

### Summary

Subcellular membrane and granule fractions derived from human platelets contain factor VIII antigen and von Willebrand factor activity but not factor VIII procoagulant activity. Circulating platelets constitute a significant reservoir of plasma factor VIII antigen, containing approximately 15% of the amount of factor VIII antigen present in platelet-poor plasma. The antibiotic ristocetin, which aggregates human platelets in the presence of von Willebrand factor, nonspecifically precipitates platelet membrane factor VIII antigen. Thus normal platelets contain surface-bound as well as internally stored von Willebrand factor, a protein synthesized by endothelial cells which is necessary for normal platelet function in vivo.

We wish to thank Dr. Peter Harpel for his helpful suggestions and Barbara Ferris for her invaluable technical assistance.

Received for publication 28 January 1975.

### References

1. Legaz, M. E., G. Schmer, R. B. Counts, and E. W. Davie. 1973. Isolation and characterization of human factor VIII (antihemophilic factor) *J. Biol. Chem.* **248**:3946.
2. Jaffe, E. A., L. W. Hoyer, and R. L. Nachman. 1973. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J. Clin. Invest.* **52**:2757.
3. Jaffe, E. A., L. W. Hoyer, and R. L. Nachman. 1974. Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1906.
4. Howard, M. A., D. C. Montgomery, and R. M. Hardisty. 1974. Factor VIII related antigen in platelets. *Thromb. Res.* **4**:617.
5. Collier, B. S., R. J. Hirschman, B. L. Kaster, and H. R. Galnick. 1973. Immunologic

- recognition of the factor VIII/von Willebrand factor (F. VIII/vwf) on the platelet surface. *Blood J. Hematol.* **42**:1021. (Abst.)
6. Nachman, R. L., A. J. Marcus, and L. B. Sofier. 1967. Platelet thrombosthenin: subcellular localization and function. *J. Clin. Invest.* **46**:1380.
  7. Marcus, A. J., D. Zucker-Franklin, L. B. Sofier, and H. L. Ullman. 1966. Studies on human platelet granules and membranes. *J. Clin. Invest.* **45**:14.
  8. Nachman, R. L., A. Hubbard, and B. Ferris. 1972. Iodination of the human platelet membrane. *J. Biol. Chem.* **248**:2928.
  9. Beckman, B., J. Flores, P. A. Witkum, and G. W. G. Sharp. 1974. Studies on the mode of action of cholera toxin. *J. Clin. Invest.* **53**:1202.
  10. Kolb, W. P., J. A. Haxby, C. M. Arroyave, and H. J. Müller-Eberhard. 1972. Molecular analysis of the membrane attack mechanism of complement. *J. Exp. Med.* **135**:549.
  11. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. *J. Biol. Chem.* **245**:3059.
  12. Hoyer, L. W. 1972. Immunologic studies of antihemophilic factor (AHF, factor VIII). IV. Radioimmunoassay of AHF antigen. *J. Lab. Clin. Med.* **80**:822.
  13. Zimmerman, T. S., O. D. Ratnoff, and A. E. Powell. 1971. Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease. *J. Clin. Invest.* **50**:244.
  14. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406.
  15. Howard, M. A., and B. G. Firkin. 1971. Ristocetin - a new tool in the investigation of platelet aggregation. *Thromb. Diath. Haemorrh.* **26**:362.
  16. Weiss, H. J., L. W. Hoyer, F. R. Rickles, A. Varma, and J. Rogers. 1973. Quantitative assay of a plasma factor deficient in von Willebrand's disease that is necessary for platelet aggregation. *J. Clin. Invest.* **52**:2708.
  17. Breckenridge, R. T., and O. D. Ratnoff. 1962. Studies on the nature of the circulating anticoagulant directed against antihemophilic factor: with notes on an assay for antihemophilic factor. *Blood J. Hematol.* **20**:137.
  18. Marcus, A. J., and M. B. Zucker. 1965. *The Physiology of Blood Platelets*. Grune & Stratton, Inc., New York. 1.
  19. Nachman, R. L. 1968. Platelet proteins. *Semin. Hematol.* **5**:18.
  20. Cooper, H. A., R. H. Wagner, and M. W. Mosesson. 1974. The cold-insoluble globulin of plasma and its relationship to factor VIII. *J. Lab. Clin. Med.* **84**:258.
  21. Weiss, H. J., J. Rogers, and H. Brand. 1973. Defective ristocetin-induced platelet aggregation in von Willebrand's disease and its correction by factor VIII. *J. Clin. Invest.* **52**:2697.
  22. Nachman, R. L., A. J. Marcus, and D. Zucker-Franklin. 1967. Immunologic studies of proteins associated with subcellular fractions of human platelets. *J. Lab. Clin. Med.* **69**:651.
  23. Weiss, H. J. 1968. Von Willebrand's disease - diagnostic criteria. *Blood J. Hematol.* **32**:668.
  24. Gore, I., M. Takada, and J. Austin. 1970. Ultrastructural basis of experimental thrombocytopenic purpura. *Arch. Pathol.* **90**:197.
  25. Holmberg, L., P. M. Manucci, I. Turesson, Z. M. Ruggeri, and I. M. Nilsson. 1974. Factor VIII antigen in the vessel walls on von Willebrand's disease and haemophilia A. *Scand. J. Haematol.* **13**:33.
  26. Tschopp, T. B., H. J. Weiss, and H. R. Baumgartner. 1974. Decreased adhesion of platelets to subendothelium in von Willebrand's disease. *J. Lab. Clin. Med.* **83**:296.
  27. Hoyer, L. W., R. P. de los Santos, and J. R. Hoyer. 1973. Antihemophilic factor

- antigen. Localization in endothelial cells by immunofluorescent microscopy. *J. Clin. Invest.* **52**:2737.
28. Levy-Toledano, S., J-P. Caen, T. Halmos, and L. Mester. 1973. Dissociation between human platelet agglomerating activity and factor VIII procoagulant activity of bovine plasma preparations by chemical treatment. I. Effect of neuraminidase. *Pathol. Biol.* **21**(Suppl.):60.
  29. Mello Perisse, A. C., J. Soria, C. Soria, and L. Mester. 1973. Dissociation between human platelet agglomerating activity and factor VIII procoagulant activity of bovine plasma preparations by chemical treatment. II. Effect of periodate oxidation. *Pathol. Biol.* **21**(Suppl.):63.
  30. Vermylen, J., M. B. Donati, G. De Gaetano, and M. Verstraete. 1973. Aggregation of human platelets by bovine or human factor VIII: role of carbohydrate side chains. *Nature (Lond.)* **244**:167.
  31. Nachman, R., and B. Ferris. 1968. Studies on human platelet protease. *J. Clin. Invest.* **47**:2530.