Immunologic Differentiation of Classic Hemophilia (Factor VIII Deficiency) and von Willebrand's Disease

WITH OBSERVATIONS ON COMBINED DEFICIENCIES OF ANTIHEMOPHILIC FACTOR AND PROACCELERIN (FACTOR V) AND ON AN ACQUIRED CIRCULATING ANTICOAGULANT AGAINST ANTIHEMOPHILIC FACTOR

THEODORE S. ZIMMERMAN, OSCAR D. RATNOFF, and ARNOLD E. POWELL

From the Departments of Medicine and Surgery, Case Western Reserve University School of Medicine, and University Hospitals of Cleveland, Cleveland, Ohio 44106

ABSTRACT Heterologous antiserum was prepared in rabbits against highly purified human antihemophilic factor (AHF, factor VIII). This antiserum blocked the clot-promoting properties of AHF and, when suitably absorbed, formed a single precipitin line against AHF upon immunoelectrophoresis. Material antigenically similar to normal AHF was detected in normal amounts in plasma concentrates in each of 22 patients with classic hemophilia, in a patient with an acquired circulating anticoagulant against AHF, and in a patient with deficiencies both of AHF and proaccelerin (factor V). AHF-like antigen was present in normal human serum. In contrast, material antigenically related to AHF was found in decreased amounts in the concentrates prepared from the plasma of 11 patients with von Willebrand's disease. The experiments described suggest that von Willebrand's disease is a disorder in which a true deficiency of AHF exists. Whether the AHF-like material found in classic hemophilia is nonfunctional through a defect in structure or through the intervention of an inhibitor has not been shown.

INTRODUCTION

Several inherited and acquired disorders are associated with functional deficiencies of antihemophilic factor (AHF, factor VIII). Conceivably, such deficiencies might reflect a decreased concentration of AHF in plasma. Alternatively, this agent might be present in essentially normal amounts, but incapable of promoting clotting, either because it has an abnormal or incomplete structure, or because it has been neutralized by some inhibitory substance. Although these alternatives have been debated for some time, their resolution has been handicapped by the unavailability of a suitable experimental approach to the problem. Recent progress in the purification of human AHF has allowed us to prepare precipitating antiserum specific for AHF. We have used this antiserum to reexamine the nature of classic hemophilia, von Willebrand's disease, congenital combined deficiencies of AHF and proaccelerin (factor V), and the circulating anticoagulant directed against AHF acquired *post partum*. In these studies, a deficiency of material antigenically related to AHF could be detected only in von Willebrand's disease. Antigen related to AHF was present in at least normal quantities in classic hemophilia, and in the other disorders tested.

METHODS

Venous blood to provide plasma for purification of AHF was withdrawn from the antecubital veins of normal human subjects, using a 17 gauge Plexitron blood-taking set (Baxter Laboratories, Inc., Morton Grove, Ill.), and was immediately added to 1/9 vol of sodium citrate buffer (pH 5.0, 0.13 M with respect to citrate) in polyethylene containers. If plasma was to be used for other purposes, blood was withdrawn with No. 18 gauge disposable needles and disposable polypropylene syringes (Monoject, Roehr Products Co., Inc., Deland, Fla.), rinsed with silicone oil [SF-

Dr. Zimmerman is a Special Research Fellow of the United States Public Health Service.

Dr. Ratnoff is a Career Investigator of the American Heart Association.

Received for publication 23 July 1970.

96(200); General Electric Co., Waterford, N. Y.], and immediately added to 1/25 vol of sodium citrate buffer (pH 5.0, 0.55 M with respect to citrate) in silicone-coated polypropylene containers. In more recent studies, plasma was prepared from blood to which 1/50 vol of 0.5 M sodium citrate buffer (pH 5.0) was added without obvious alteration in our results. Plasmas were obtained from normal individuals, patients with classic hemophila, von Willebrand's disease, or combined deficiencies of AHF and proaccelerin, and from a patient with a circulating anticoagulant directed against AHF, first detected *post partum*.

Platelet-deficient plasma for AHF purification was separated from whole blood by centrifugation at 2°C in a Servall Automatic Superspeed Refrigerated Centrifuge, RC-2 (Ivan Sorvall, Inc., Norwalk, Conn.) at 16,000 *g* for 30 min. The plasma was withdrawn and respun at 16,000 *g* for 15 min. Plasma for other purposes was separated from whole blood by centrifuging at 2°C in an International Refrigerated Centrifuge, PR-2 (International Equipment Co., Needham Heights, Mass.) at 3500 *g* for 15 min. Plasma for AHF purification was used immediately. Plasma for other purposes was assayed for AHF activity immediately and stored at −70°C in silicone-coated polyethylene containers.

Crude AHF concentrates for AHF purification were prepared from approximately 140 ml of normal plasma by a modification of Johnson's method (1). At −3°C, sufficient 53.3% ethanol was added to bring the final concentration of ethanol to 3%. The plasma was stirred gently for 30 min and then sedimented at 16,000 *g* for 10 min at −3°C. The supernatant solution was discarded, and the gummy precipitate was dissolved in 30 ml of imidazole-saline buffer (0.02 M imidazole, 0.14 M NaCl, pH 6.5) at 37°C. An equal volume of 20% (w/v) polyethylene glycol (polyethylene glycol 6000, average mol wt 6000-7500; Matheson, Coleman, & Bell, East Rutherford, N. J.) in imidazole-saline buffer was then added at room temperature. The mixture was divided into four equal aliquots, and the white precipitate which formed was sedimented at 12,000 *g* for 10 min at room temperature. Each precipitate was then suspended in 5.0 ml of cold 6% ethanol and recentrifuged, and the supernatant fluid was discarded. The precipitates were tough and gummy. Each was dissolved in 2 ml of imidazole-saline buffer at room temperature over a period of 1–2 hr, stirring gently with a magnetic stirrer. The four aliquots were then combined and sedimented at 12,000 *g* for 10 min at room temperature to remove any remaining particulate material.

Crude ethanol concentrates of AHF for immunologic studies were prepared at −3°C in silicone-coated glass centrifuge tubes from 2.5 or 5.0 ml of citrated plasma to which was added sufficient 53.3% ethanol to achieve a final concentration of 3%. The mixture was allowed to stand for 30 min. The precipitate was separated by centrifugation at −3°C for 10 min at 12,000 *g*. The supernatant plasma was saved for absorption of antiserum (see below), and the precipitate was dissolved in 0.2 ml of electrophoresis buffer (0.05 M sodium barbital buffer at pH 8.4) or in diluted antiserum (see below). Recovery of AHF activity in these ethanol concentrates was tested in 10 normal plasma samples; recovery averaged 48.3% with a range of 39%–57% and a standard deviation of 5.5%. Since purified AHF has a specific activity 8000-fold (or more) greater than that of plasma (see below), the maximal amount of this clotting factor is about 10 μg/ml of plasma. Ethanol concentrates, then, probably contained a maximum of 125 μg/ml, when prepared from 5 ml of plasma, and 62.5 μg/ml, when prepared from 2.5 ml of plasma. Protein determinations were obtained in trial experiments and were not performed on fractions used for immunologic studies for want of sufficient material.

Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden), a 4% agarose gel with a particle size of 40–190 μ in the swollen state, was suspended in imidazole-saline buffer. Columns of the gel, 5.0 cm in diameter by 45.0 cm in height, were supported by nylon netting. The column was washed at 4°C with a constant flow of the same buffer. The recovery of AHF was greatly improved by pretreating the column with an ethanol-precipitated crude concentrate of outdated plasma. Pretreatment with whole plasma, as previously described (2), resulted in spontaneous clotting in the column bed, presumably due to the different pH used in this set of experiments.

Highly purified AHF was prepared by gel filtration through the Sepharose 4B column by applying 8 ml of crude concentrate at room temperature and eluting with imidazole-saline buffer at a pressure of 80–120 cm of water above the outlet of the column. The column and the eluting buffer were at 4°C. The effluent which constituted the void volume was collected in 5.0 ml aliquots and assayed immediately for AHF activity and total protein. The contents of the tubes with highest activity, containing approximately 1 U of AHF activity and 10 μg/ml of protein or less, were combined, and AHF was stabilized by the addition of sufficient solid glycine hydrochloride to make a 2 M solution. The addition of glycine stabilized AHF activity in this diluted solution at 50-70% of the original activity upon storage at 4°C for 2–3 days. The solution, usually 20–40 ml, was concentrated to 0.2–0.5 ml by pressure dialysis against an Amicon PM-30 Diaflo membrane in the Amicon 8-MC Micro-Ultrafiltration System (Amicon Corp., Lexington, Mass.). As much as 50% of AHF activity was lost during concentration. Most of the loss appeared to be the result of adhesion of the protein to the membrane; this could be minimized by the use of low pressure (less than 10 psi) and by allowing time for eluting material from the membrane after pressure was released. The concentrated preparations were estimated to contain a maximum of 0.2–1.0 mg of AHF per ml.

Rabbit antibody to highly purified human AHF was prepared by injecting a mixture of equal parts of higly purified AHF, containing approximately 200 μg of protein, and alumina C γ-Gel (A grade, Calbiochem, Los Angeles, Calif.), into the foot pads and toes pads of white New Zealand female rabbits. 3 wk later, the same amount of antigen in alumina was reinjected in the same manner, and 6 days thereafter blood was drawn from a leg vein and allowed to clot in glass tubes. The blood was kept at 37°C overnight and then centrifuged at 12,000 *g* for 10 min.

Antiserum prepared in this way contained antibodies against several plasma proteins (Fig. 1). The antiserum was therefore absorbed with an AHF-poor fraction of human plasma. A precipitate was prepared at −3°C from the supernatant plasma after removal of the fraction precipitated by 3% ethanol, as described previously, by the addition of sufficient 53.3% ethanol to bring the final ethanol concentration to 8%. The mixture was gently stirred for 30 min. The precipitate (which was relatively depleted of AHF) was sedimented at 12,000 *g* for 10 min at −3°C and dissolved in a volume of antiserum equal to twice the initial plasma volume. The mixture was allowed to incubate overnight at 37°C and the resulting fibrin clot and precipitate were removed by centrifugation at 12,000 *g* for 10 min. The antibody solution was then heated at 60°C for 60 min. After the *absorbed antiserum* had cooled to room

TABLE I

The Effect of Anti-AHF Antiserum on AHF Activity

Mixture	AHF activity
	U/ml
Normal rabbit serum + normal plasma	0.50
Unabsorbed antiserum + normal plasma	0.01
Absorbed antiserum + normal plasma	0.02

temperature, 10 mg of tricalcium phosphate was added to each milliliter of absorbed serum, the mixture agitated for 10 min at room temperature, and the treated serum separated by centrifugation at 12,000 *g* for 10 min. Antiserum used only for immunodiffusion studies was not calcium phosphate-absorbed or heated. The volume of precipitate needed to absorb contaminants must be determined experimentally for each lot of antiserum.

Testing of antiserum for anti-AHF activity was performed by incubating an appropriate dilution of antiserum in barbital-saline buffer (0.025 M barbital-sodium barbital, 0.125 M NaCl, pH 7.5) with normal human plasma for 2 hr at 37°C and then assaying the mixture for AHF activity. Similarly treated and diluted serum from an unimmunized rabbit, mixed with the human plasma, was used as the control. The antiserum was usually capable of inactivating about one-half the AHF of an equal volume of normal plasma when antiserum was diluted 16-fold. The relationship between the concentration of antiserum and the degree of inactivation of AHF was not linear, and even undiluted antiserum did not completely inactivate all AHF in an equal volume of plasma (Table I).

Screening of antiserum for anticlotting activity was accomplished by mixing undiluted antiserum with equal parts of fresh normal human plasma and incubating for 2 hr at 37°C. The mixture was then assayed directly for Christmas factor (factor IX) activity. The partial thromboplastin time, one-stage prothrombin time, and thrombin time were measured. As a control, similarly treated serum from a normal rabbit was mixed with an equal part of the normal human plasma.

Blocking of the anti-AHF activity of antiserum was performed with concentrates of plasma. Crude ethanol concentrates of plasma to be tested, normal or abnormal, were prepared from 5 ml of plasma and dissolved in 0.5 ml of absorbed antiserum which had been diluted appropriately, usually 8-fold, with barbital-saline buffer. A control of 0.5 ml of antiserum to which no concentrate was added was also carried through the following steps. The mixture was allowed to incubate for 2 hr at 37°C and then incubated for 1 hr at 60°C. No AHF activity could be detected at this point. The mixture was centrifuged at 12,000 *g* for 10 min, and 0.40 ml of supernatant solution was pipetted into 10 × 75 mm polystyrene tubes and allowed to sit at 2°C overnight. A volume of 0.05 ml of fresh plasma was then added to the blocked antibody, which was incubated for 2 hr at 37°C. Sufficient barbital-saline buffer was then added to achieve a 20-fold final dilution of the fresh plasma, and the mixture was assayed for AHF activity in clotting assays. The results of such tests are expressed in units of residual AHF less the residual units in a control to which no blocking concentrate was added, 1 U being the functional activity found in 1 ml of a pool of 25 normal human plasmas (see below).

For each experiment, in order to estimate the capacity of the ethanol concentrates tested to block the antiserum, a calibration curve was constructed by preparing 3% ethanol concentrates from 5.0, 2.5, 1.25, 0.6, and 0.3-ml aliquots of standard pooled normal plasma. Each precipitate was dissolved in 0.5 ml of antiserum at the same concentration used for the study of normal and abnormal plasmas. A linear relationship existed between the clotting time obtained in the AHF assay and the logarithm of the volume of plasma used to prepare these ethanol concentrates. A linear relationship also could be drawn between the logarithm of the number of units of residual AHF in the mixture of antibody and the ethanol concentrates and the logarithm of the volume of plasma used to prepare the concentrates. The logarithmic nature of these plots magnified the error in measuring blocking by small amounts of antigen. The conditions described for comparing blockage of antibody by different plasmas were selected to minimize this error. If antibody concentration was too high, no blocking could be demonstrated; if too low, insufficient differences were obtained between blocking with varying amounts of antigen. Incomplete blocking occurred if the material was incubated at room temperature or on ice. Inadequate time for the initial period of incubation, including time after heat inactivation, prevented adequate blocking, whereas too much time spent in the final incubation resulted in excessive nonspecific inactivation of AHF.

In a typical experiment for determination of appropriate condition, the residual AHF activity in a mixture of standard plasma and antiserum was 0.07 U/ml. When the antiserum was previously absorbed with the ethanol concentrate of 5 ml of normal plasma, the residual AHF activity in the standard plasma was 0.30 U/ml. Residual AHF activity after the antiserum had been absorbed with the concentrate of 0.3 ml of normal plasma was 0.13 U/ml, while intermediate amounts of the ethanol concentrate blocked intermediate amounts of antibody.

The number of samples tested in each run was limited because the natural lability of AHF blurred differences in blocking if too much time elapsed between AHF assay of the first and last samples. It must be emphasized that normal and abnormal plasmas were tested under identical conditions.

Agarose gel for immunodiffusion and immunoelectrophoresis studies was prepared by dissolving agarose (B grade; Calbiochem) at a concentration of 0.9% (w/v) in barbital buffer (0.05 M sodium barbital, pH 8.4) containing 0.1% sodium azide as a preservative. Agar could not be used because the antigen moved poorly in this medium. 2½ ml of agarose in buffer, at 90°C, were applied to alcohol-cleaned 1 inch by 3 inch microscope slides and allowed to cool. Wells for immunodiffusion were cut by hand with a 3 mm punch and were 2–3 mm apart. Wells and troughs for immunoelectrophoresis were cut with dies.

In immunodiffusion studies of whole plasma, the antigen wells were refilled 3 or 4 times prior to complete emptying. This was not necessary when ethanol concentrates of plasma or concentrated purified AHF were used as the antigen.

Immunoelectrophoresis was performed on ethanol concentrates and concentrated purified AHF, using a constant potential of 450 v for 1 hr at room temperature. To determine relative electrophoretic mobility, antiserums against human β_{1C}-β_{1A}, or α_2-macroglobulin were tested simultaneously with anti-AHF antiserum, against a 3% ethanol concentrate of plasma. The wells contained 5 μl of sample, and the troughs were filled with 0.2 ml of undiluted anti-

serum. Thus the wells were estimated to contain at most 0.625 μg of AHF in experiments with crude ethanol concentrates, and 1–5 μg of AHF in experiments with purified AHF.

Quantitative immunoelectrophoresis of AHF was modified from the method of Laurell (3). A volume of 0.3 ml of absorbed antibody was added to 6.5 ml of 0.9% agarose in barbital buffer (0.05 M sodium barbital, pH 8.4) at 56°C, and the mixture was allowed to gel on a clean, leveled 2 inch by 3 inch microscope slide. Ten μl of each solution of antigen were placed in wells 3 mm in diameter, using Oxford sampling pipettes with disposable plastic tips (Oxford Laboratories, San Mateo, Calif.) and a constant current of 6 ma per slide applied for 18 hr at room temperature through wicks of Whatman No. 541 filter paper. The initial voltage was approximately 350 v. The antigen used was a crude ethanol concentrate of 2.5 ml of plasma, dissolved in 0.2 ml of electrophoresis buffer, and thus contained a maximum of 12.5 μg of AHF in 0.2 ml. The antigen appeared to move slowly and the prolonged time was necessary to achieve satisfactory movement. Assays were performed with duplicate crude ethanol concentrates. In experiments in which normal amounts of antigen were anticipated, antigen was tested undiluted and diluted twofold in order to bring its concentration within the range of the standard curve. The results obtained at different dilutions were averaged. When subnormal amounts of antigen were anticipated, the concentrates were tested only undiluted. Duplicate determinations of antigen concentration in crude concentrates varied by as much as 25% but were generally within 10% of each other.

A standard curve was constructed by testing doubling dilutions prepared from ethanol concentrates of a standard frozen plasma pool (see below) and plotting the results on logarithmic coordinates; a standard curve was run on each slide. Antigen could be detected when the ethanol concentrate was diluted 16-fold, that is, so that the concentration of antigen was estimated to be about 4 μg of AHF per ml or less. The amount of antigenic material present in ethanol concentrates of the pooled plasma was arbitrarily defined as 1.0 antigen U/ml. In some experiments, such as that depicted in Fig. 3, the standard concentrate was prepared from 5.0 ml of plasma and then diluted serially to allow estimation of values above 1.0 U/ml. When unconcentrated plasma (containing an estimated maximum of 10 μg of antigen per ml) was tested directly, the test was not sensitive enough to allow detection of antigen below 25% of the standard, and at higher concentrations of antigen in plasma, duplication of results was often unsatisfactory. The sensitivity of quantitative immunoelectrophoresis for measurement of AHF was slightly greater than that originally reported for the detection of albumin, a much smaller molecule, by Laurell (3), who measured 0.5–5 μg of protein readily.

Assays of AHF and Christmas factor were performed by a modification of the partial thromboplastin time described earlier (4). The partial thromboplastin time, prothrombin time, and thrombin time techniques have been summarized elsewhere (5).

Rabbit anti-human fibrinogen antiserum, goat anti-β_{1C}-β_{1A}-globulin antiserum, and goat anti-α_2-macroglobulin antiserum were obtained from Hyland Laboratories, Los Angeles, Calif.

An AHF standard plasma was prepared from pooled plasma freshly drawn from 25 normal white adult male subjects between the ages of 21 and 40 yr. The pool was frozen in small aliquots in silicone-coated polyethylene containers at −70°C within 2 hr of venepuncture and stored at this temperature. The procoagulant AHF activity of a frozen aliquot was immediately determined in comparison with that of an unfrozen sample of the pooled plasma, said arbitrarily to contain 1.0 U of AHF activity per ml (4). The AHF activity in the frozen pooled plasma was 0.86, 0.92, and 1.0 U/ml in three different preparations. It was relatively stable for 2 months, during which time approximately 15% of activity was lost. As already noted, the amount of AHF-like antigenic material in ethanol concentrates of this pooled plasma was defined as 1.0 antigen U/ml. *This arbitrary unitage is independent of that used to quantify functional AHF; there is no reason to assume that the unitages of functional and antigenic AHF are identical.*

The effect of a specific circulating anticoagulant on the AHF-like antigen was tested by incubating a 1:20 dilution of the anticoagulant plasma in normal plasma. The mixture was incubated for 2 hr at 37°C and assayed for AHF activity; an ethanol concentrate was made, and AHF-like antigen concentration was measured by immunoassay. A concentrate from plasma to which no anticoagulant had been added was used as a control.

Protein concentration was determined by the method of Lowry, Rosebrough, Farr, and Randall (6).

RESULTS

Properties of a rabbit antibody against human AHF. AHF, prepared from human plasma by the technique described, was purified 6000–16,000-fold compared with the plasma from which it had been separated. Only preparations at least 8000-fold purified were used in the experiments to be described.

Upon immunoelectrophoresis against unabsorbed rabbit anti-AHF serum, preparations of purified AHF which had been concentrated 40–80-fold formed a single line in the β-globulin region (Fig. 1*A*). Nonetheless, the AHF was not pure, since antiserum prepared against it formed multiple lines when tested by immunoelectrophoresis against 3% ethanol concentrates of normal human plasma (Fig. 1*B*, top). The same antiserum, absorbed with an AHF-depleted fraction of plasma, formed only a single line against the 3% ethanol concentrate (Fig. 1*B*, bottom). Similarly, as anticipated from the preceding experiment, it formed a single line against highly purified AHF. Upon immunoelectrophoresis, no precipitate appeared between normal plasma and the absorbed anti-AHF antiserum, as if normal plasma did not contain enough antigen for its detection. A single line was observed on simple gel diffusion when the well containing plasma was refilled several times. This line formed a reaction of identity with the single line from a 3% ethanol concentrate of plasma and highly purified AHF (Fig. 2*A*).

In Fig. 1*A, B,* and *C,* the AHF-like antigen lines appear to differ slightly in their mobilities. Since the slides were prepared on different days, the variations are probably not significant.

The absorbed antiserum retained essentially all of the anti-AHF activity of the unabsorbed antiserum (Table I). It lengthened the partial thromboplastin time of normal plasma, but it did not inactivate Christmas factor or lengthen the prothrombin time or thrombin time of normal plasmas with which it was mixed (Table II). The absorbed antiserum did not form a precipitin line against human fibrinogen, as tested by immunoelectrophoresis. The antibody was still active after heating at 60°C for 60 min and adsorption with calcium phosphate.

Studies of individuals with disorders of blood coagulation. A reaction of identity was observed on double gel diffusion between 3% ethanol concentrates of hemophilic and normal plasma (Fig. 2*A*). The immunoelectrophoretic mobility of the antigen of hemophiliacs was similar to that of normal individuals (Fig. 1*C*), although a small difference in mobility was not excluded. This experiment suggested that hemophilic plasma might contain antigenic material identical with AHF. Further evidence was provided by the observation that rabbit anti-AHF activity was blocked to a comparable degree by 12 normal and 14 hemophilic plasma concentrates (Table III). Plasma concentrates from these individuals also contained similar amounts of AHF-like antigen when measured by quantitative immunoassay. Hemophiliacs with circulating anticoagulants directed against AHF could not be tested in the blocking system because some of the anticoagulant remained in the ethanol concentrates.

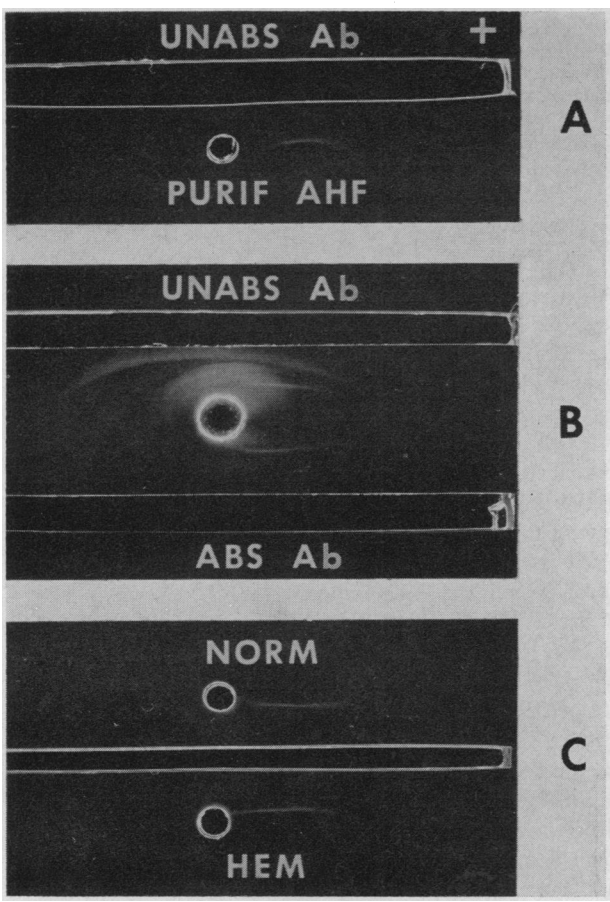

FIGURE 1 Immunoelectrophoresis of different preparations of AHF developed against absorbed and unabsorbed antiserum. *A:* The well contained highly purified AHF and the trough contained unabsorbed antiserum. *B:* The well contained ethanol concentrates of normal plasma, the upper trough unabsorbed antibody, the lower trough absorbed antibody. *C:* An ethanol concentrate of plasma from a normal individual was placed in the upper well, a concentrate from a hemophiliac in the lower well, and absorbed antiserum in the trough. In each experiment, the anode was at the right.

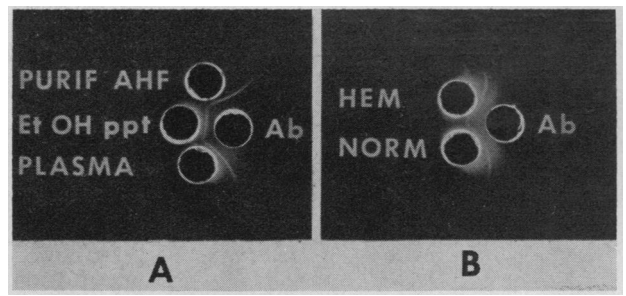

FIGURE 2 Double gel diffusion with various AHF preparations. *A:* The left upper well contained highly purified AHF, the left middle well an ethanol concentrate of normal plasma, and the left lower well normal plasma. *B:* The left upper well contained a concentrate of normal plasma, the left lower well a concentrate of hemophilic plasma.

22 patients with hemophilia (members of 21 families) and 49 normal individuals (22 females and 27 males) had similar quantities of antigen in ethanol concentrates of their plasma, as measured by the Laurell technique (Fig. 3). The arithmetic mean concentration of antigen in the normal individuals was 0.95 antigen U/ml (SD = 0.35). The mean concentrations of antigen were not

TABLE II
The Effect of the Anti-AHF Antiserum on Blood Coagulation

Test	Clotting Time: Normal rabbit serum + normal plasma	Clotting Time: Anti-AHF serum + normal plasma
	Sec	*Sec*
Partial thromboplastin time	100.3	255.4
Christmas factor assay	67.9	68.7
Thrombin time	32.2	31.6
Prothrombin time	16.6	16.8

significantly different in normal and hemophilic subjects. The mean concentration of antigen in normal male subjects (0.88 antigen U/ml; SD = 0.31), however, was significantly different from that of the hemophiliacs, at the 5% level. Although this difference may have been due to a difference in electrophoretic mobility between normal and hemophilic antigens, no such difference was noted upon immunoelectrophoresis in agarose gels.

The concentration of antigen in ethanol concentrates of the plasma of hemophiliacs with circulating anticoagulants was within the normal range. No experiments were performed to test whether the ethanol precipitation technique dissociated complexes of circulating anticoagulant and AHF, but the precipitate contained appreciable amounts of anticoagulant. In normal individuals, AHF procoagulant activity was a linear function of the concentration of antigen, as measured immunologically (Fig. 4). In contrast, the functional AHF activity in hemophilic plasma bore no relationship to the concentration of antigen detected.

Von Willebrand's disease is a bleeding disorder in which AHF activity often is decreased. In 11 patients with von Willebrand's disease (six males and five females, members of eight families), antigen was decreased considerably as compared with both the normal and hemophilic population (Figs. 3 and 4). In blocking assays, concentrates of the plasmas of all von Willebrand's disease patients blocked the antibody significantly less than did either normal or hemophilic samples (Table III). As in normal individuals, functional AHF activity appeared to be a function of the apparent antigen content in the ethanol concentrates from plasmas of patients with von Willebrand's disease (Fig. 3). In one patient, the concentration of antigen rose more than three times (from 0.07 to 0.22 antigen U/ml) during pregnancy, while functional activity rose proportionately (from 0.14 to 0.58 U/ml). All of the von Willebrand's patients appeared to fall within the 95% confidence belts of a downward extension of the plot of activity against antigen concentration for normal individuals (Fig. 4). The amount of antigen present in ethanol precipitates of von Willebrand's plasma was usually too low to be detected by immunoelectrophoresis. In one case, a line was visible when the precipitate was twice concentrated compared to the usual procedure; migration of this antigen appeared to be the same as for the normal antigen, although a small difference may not have been detectable. In another case, a faint line was visible by the usual procedure, but the precipitate was too small to be certain that its migration was identical with that of normal AHF.

It is possible that the observed difference between hemophilic and von Willebrand's plasma concentrates may have been due to a difference in ethanol precipitability of antigen in the two diseases. To test this, quantitative immunoelectrophoresis was performed on the unconcentrated plasma of 13 hemophiliacs and 10 patients with von Willebrand's disease. This assay was less reproducible when run on unconcentrated plasma and was not sensitive enough to detect AHF-like antigen in concentrations less than 12–25% of normal. Nevertheless, the results were qualitatively similar to those obtained on plasma concentrates. Two of the patients with von Willebrand's disease had concentrations of antigen of approximately 40% while the rest were less than 25% of the standard normal plasma. Of the hemophiliacs, one had a concentration of 25%, and the rest were between 50% and 200% of the standard normal plasma.

One individual with congenital combined deficiencies of AHF and proaccelerin (7) was tested. AHF-like antigen was within the normal range (0.70 antigenU/ml), although AHF activity was only 0.06 U/ml. Concentrates of this patient's plasma blocked the antibody to a degree comparable with normal and hemophilic plasma fractions.

One patient who had a potent circulating anticoagulant directed against AHF, detected after pregnancy, was tested. Normal quantities of antigen were found in her plasma concentrates (1.20 antigen U/ml). On immunoelectrophoresis, the mobility of the patient's AHF-like antigen was indistinguishable from that of normal AHF. Because of the presence of the anticoagulant, the blocking effect of her plasma concentrates on the anti-AHF activity of the antibody could not be determined in blocking assays.

Some observations on the AHF-like antigen. Heating purified, glycine-stabilized AHF at 60°C for 90 min did not alter the concentration of antigen as determined by immunoelectrophoresis, although functional activity decreased by more than 90%.

Antigen was demonstrated by quantitative immunoelectrophoresis in normal human serum in approximately the same concentration as in normal plasma. Insufficient AHF-like antigen was present in normal plasma or serum for routine immunoelectrophoresis.

The addition to normal plasma of 1/20 part of circulating anticoagulant plasma and incubation for 2 hr at 37°C resulted in complete inactivation of AHF. Nevertheless, the amount of antigen detected by quantitative immunoelectrophoresis in ethanol concentrates was not altered significantly.

DISCUSSION

The experiments reported here examined whether the plasma of patients with classic hemophilia contain nonfunctional material antigenically related to AHF. Such plasmas were compared with those of patients with von Willebrand's disease and other disorders of AHF func-

TABLE III

Comparison of AHF-Like Antigen by Antiserum Blocking and Quantitative Immunoelectrophoresis

Subject	Plasma AHF activity	Antiserum blocking*: Residual AHF activity	Quantitative immunoelectrophoresis: AHF-like antigen
	U/ml	*U/ml*	*U/ml*
Normal			
He	1.20	0.14	0.91
Ha	1.46	0.17	1.34
Mc	1.15	0.24	1.00
Fo	0.86	0.14	0.90
Ge	1.12	0.19	0.86
Sh	0.92	0.15	0.64
Te	1.29	0.16	1.16
Mu	1.72	0.17	1.60
Al	1.36	0.26	1.32
Bu	0.72	0.21	0.67
Ge	0.72	0.13	0.50
Si	0.50	0.10	0.35
Arithmetic Mean	1.09	0.17	0.93
SD	0.35	0.05	0.37
Hemophiliacs			
Ma	<0.01	0.14	0.84
Sh	<0.01	0.15	0.72
Ta	0.28	0.27	1.20
Ju	<0.01	0.09	1.52
Ma	<0.01	0.09	1.32
Nu	<0.01	0.10	0.50
Ry	0.13	0.17	1.30
Sa	<0.01	0.23	1.60
Zi	<0.01	0.21	1.60
Ki	<0.01	0.12	0.62
Bo	<0.01	0.13	0.90
Mi	0.06	0.17	0.80
Ge	0.03	0.16	0.80
Sc	<0.01	0.18	0.88
Arithmetic Mean	0.04	0.16	1.04
SD	—	0.05	0.37
Von Willebrand's Disease			
Ir	0.14	0.09	0.07
Bu	0.08	0.04	0.00
D. Mc	0.38	0.08	0.12
L. Mc	0.37	0.02	0.15
M. Ec	0.28	0.03	0.13
B. Ec	0.28	0.00	0.04
Gr	0.28	0.02	0.18
Ki	0.30	0.00	0.06
Ch	0.28	0.03	0.16
Fe	0.08	0.05	0.09
Arithmetic Mean	0.23	0.04	0.10
SD	0.13	0.03	0.05
Combined AHF and proaccelerin deficiency			
La	0.05	0.09	0.70

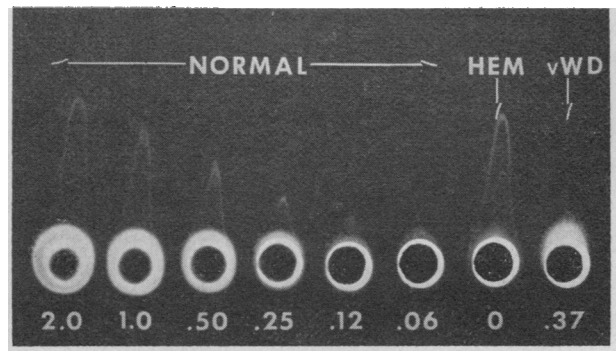

FIGURE 3 Quantitative immunoelectrophoresis of AHF-like antigen. The first six wells contained serial dilutions of an ethanol concentrate prepared from 5 ml of a standard plasma pool, the seventh well concentrate prepared from 2.5 ml of hemophilic plasma, and the last well, the concentrate from 2.5 ml of plasma from an individual with von Willebrand's disease. The corresponding functional AHF *activities* (U/ml) are indicated beneath each well.

tion. Our studies took advantage of the availability of heterologous antiserum directed specifically against human AHF.

The antigen used to induce antibody formation was highly purified and usually formed only a single precipitin line when tested by immunoelectrophoresis against unabsorbed antibody. The AHF preparations used for immunization did contain traces of other antigens, since unabsorbed antibody formed multiple precipitin lines against a crude ethanol precipitate of normal plasma. Antibodies against these contaminants were removed by absorption with a suitable fraction of normal plasma. The absorbed antiserum contained a single precipitating antibody, directed against the predominant antigen in purified preparations of AHF, and it specifically inhibited the clot-promoting function of AHF. These two properties were probably due to the same antibody since the AHF activity of plasmas varied consistently with the

In comparing normal individuals to hemophiliacs, no significant differences were detected in arithmetic mean residual AHF, after blocking of antibody by plasma concentrates, or in arithmetic mean AHF-like antigen concentrations. In contrast, the mean residual AHF and AHF-like antigen concentrations in von Willebrand's disease differed from those of normal and hemophilic subjects at the 5% level of significance. A multiple comparion test (37) was used.

The coefficient of correlation between mean residual AHF and AHF-like antigen concentrations for all values was 0.75, significant at less than the 1% level.

* The results of experiments in which antiserum was blocked by AHF-like antigen are expressed in units of AHF activity remaining in a standard plasma which was added to the blocked antiserum. The amount of AHF activity left in the standard plasma is a measure of the extent to which the antibody has been blocked.

amount of antigen in AHF-rich concentrates of the same plasmas, as detected by quantitative immunoelectrophoresis. Further, when plasma concentrates from normal individuals, hemophiliacs, and individuals with von Willebrand's disease were viewed as groups, their ability to block the antibody's clot-inhibitory properties correlated with their content of precipitating antigen. These findings suggest that the precipitating antibody in our antiserum was directed specifically against AHF.

Using this specific antibody, we demonstrated normal amounts of material antigenically related to AHF in the plasma of each of 22 patients with classic hemophilia, as tested by quantitative immunoelectrophoresis. The validity of this conclusion depends upon the similarity between the rate of migration of antigen in normal and hemophilic plasma concentrates, as demonstrated by immunoelectrophoresis. Further, in each of 14 patients, the plasma had a normal capacity to neutralize the antiserum's specific clot-inhibitory properties. The amount of antigen detectable in the plasma of hemophiliacs did not correlate with the severity of their disease, which was related to the titer of functional AHF.

Patients with von Willebrand's disease, on the other hand, had markedly diminished AHF-like antigen in their plasma concentrates as demonstrated both by quantitative immunoelectrophoresis and by neutralization of the antibody's clot-inhibitory properties. It is unlikely that a difference in ethanol precipitibility of the AHF-like antigen is responsible for these findings as similar results were obtained when quantitative immunoelectrophoresis was performed on unconcentrated plasma. These observation are in agreement with the view that a true deficiency of AHF exists in von Willebrand's disease.

The experiments described demonstrate that some form of AHF is synthesized in classic hemophilia. They do not differentiate among several possible explanations for the functional inactivity of this clotting factor. Perhaps patients with hemophilia synthesize a nonfunctional type of AHF, either abnormally or incompletely formed, but

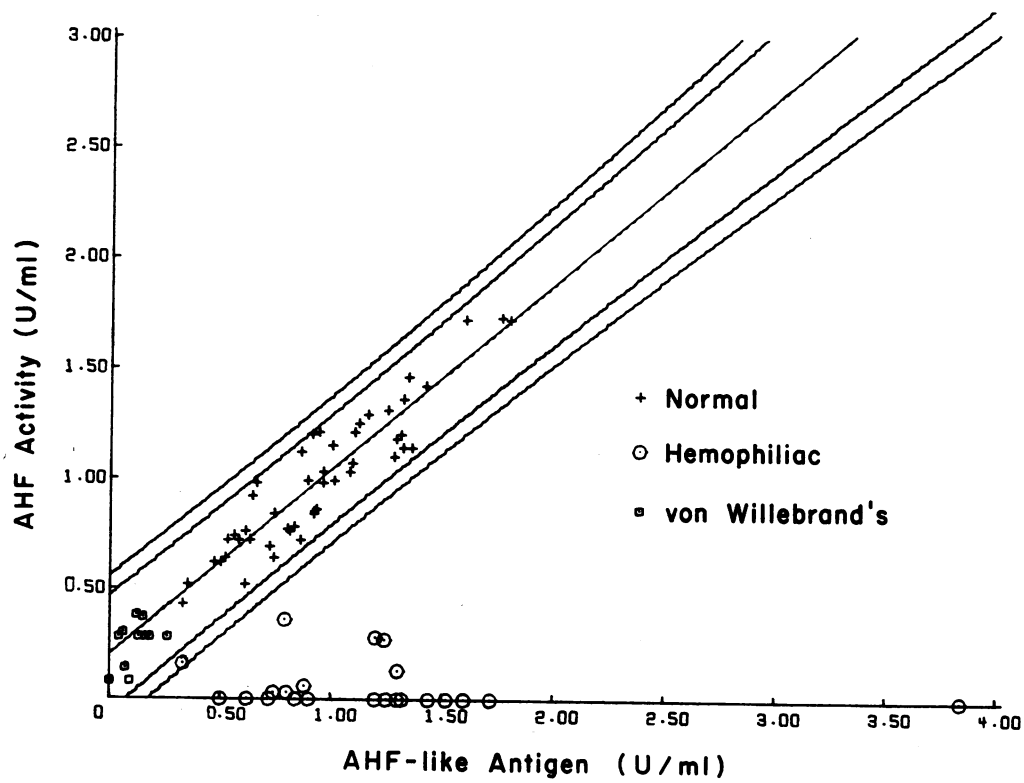

FIGURE 4 The relationship of AHF activity to AHF-like antigen in normal individuals hemophiliacs, and individuals with von Willebrand's disease. AHF-like antigen was measured by quantitative immunoelectrophoresis. The center line is the regression line for normal individuals, the outermost lines represent the 99% confidence belt, and the other two lines the 95% confidence belt. The equation for the regression line was $y = 0.200 + 0.840x$, where y is AHF activity in U/ml, and x is the concentration of antigen in antigen U/ml; the standard deviation from regression was 0.123. The intercept of the normal regression line with the vertical axis was not significantly displaced from the origin.

immunochemically related to normal AHF. Alternatively, as Tocantins (8) proposed many years ago, classic hemophilia may be due to the suppression of AHF activity by inhibitory agents. The AHF activity of the plasma of patients with von Willebrand's disease increases disproportionately after the transfusion of either hemophilic or normal plasma (9–11). One possibility, then, is that the antigenic material present in the plasma of hemophiliacs is an incomplete form of AHF, and that synthesis or the addition of appropriate cofactors is completed by the patient with von Willebrand's disease. Notably, this construction does not assume that all of the antigenic material found in normal plasma is functional. Alternatively normal or hemophilic plasma might activate a precursor of AHF in the von Willibrand's disease patient which is immunochemically dissimilar to normal or hemophilic AHF-like antigen.

Our hypotheses are by no means satisfying, but it is interesting to note that in another situation, Fabry's disease, the transfusion of normal plasma is followed by a rise in the concentration of an enzyme, ceramide trihexosidase, present in diminished amounts in this disorder (12). As with AHF in von Willebrand's disease, the rise is considerably greater than calculated from the amount infused.

The results we have obtained were foreshadowed by the work of others. Using physicochemical methods, Hershgold, Silverman, Davison, and Janszen (13) and Barrow and Graham (14) reported the isolation of material similar to AHF from hemophilic plasma. Heterologous antisera directed against relatively crude AHF preparations have been prepared by several investigators (15–20). Utilizing such heterologous antisera, Shanberge and Gore (16), Berglund (17), and Piper and Schreier (19) demonstrated that the plasma of hemophiliacs neutralized the capacity of the antisera to inactivate AHF. Uszynski (20), on the other hand, found neutralizing antigen only in the plasma of patients with moderately severe classic hemophilia, and then only inconstantly. Neither patients with severe hemophilia nor von Willebrand's disease had detectable neutralizing antibody. Similarly, Bennett (21), using an immunoassay and heterologous antibody, detected functionally inactive AHF-like antigen in some hemophiliacs but not in others. Perhaps differences in technique account for the differences between our results and those of Uszynski and Bennett. We found all of the steps outlined in Methods to be necessary for meaningful comparison of plasma samples.

Uszynski (22) reported the presence of a precipitating antigen in normal plasma and in the plasma of 43 of 50 hemophiliacs; this antigen was detected in diminished amounts in the plasma of six of eight patients with von Willebrand's disease. He did not correlate the presence of this antigen with the capacity to block anti-AHF antibody nor did he demonstrate its presence in a highly purified AHF preparation.

Vainer and Caen (23), using a heterologous antibody prepared against whole plasma, also described an antigen in normal and hemophilic plasma which was not present in von Willebrand's disease. The mobility of the antigen was that of a γ-globulin, grossly different from that which we observed. They did not report inactivation of AHF by the antibody.

Other investigators have approached the question of whether patients with classic hemophilia synthesize nonfunctional AHF by using homologous circulating anticoagulants against this clotting factor instead of heterologous antibodies. Goudemand, Foucaut, Huting, and Parquet-Gernez (24) found material in normal and hemophilic plasma which blocked the capacity of two potent circulating anticoagulants to inactivate AHF. No such blocking properties were formed in von Willebrand's disease. One of Goudemand's circulating anticoagulants formed a precipitate against normal and hemophilic plasmas. The material precipitated possessed different electrophoretic properties than the antigens described here. Other circulating anticoagulants have not possessed precipitating qualities. Abilgaard et al. (25), Hoyer and Breckenridge (26), Denson et al. (27), and Feinstein et al. (28) employed circulating anticoagulants developing spontaneously in otherwise normal individuals. With the exception of Abilgaard (25), who studied a smaller group of patients, all these investigators demonstrated antigenic material in a small proportion of hemophiliacs and none in von Willebrand's disease.

The explanation for the differences found with circulating anticoagulants and heterologous antibodies is not clear at this time. That circulating anticoagulants are blocked by plasma obtained from a minority of patients with classic hemophilia, while rabbit antiserum was inhibited by all plasma we tested, suggests that the heterologous antiserum attaches to sites other than those attacked by the anticoagulants. Corroborative evidence for this has been obtained in incomplete, unpublished studies using mammalian plasma.[1] Human circulating anticoagulants inactivated AHF activity in bovine, sheep, goat, horse, rabbit, hamster, guinea pig, chimpanzee, cat, dog, and pig plasmas. In contrast, the rabbit antiserum was ineffective against rabbit and hamster and had little effect upon bovine AHF. Thus, the antiserum behaved as if it acted upon different antigenic determinants than those of human AHF. These results, similar to those published by others (15, 16, 29) must be accepted only in a tentative way, since it is possible that extraneous factors influenced the results.

[1] Zimmerman, T. S. Unpublished data.

During the clotting process, AHF activity disappears from plasma, at least in part through the action of thrombin (30). Thrombin in low concentrations has been shown to potentiate the clot-promoting properties of AHF, and in high concentrations it inactivates this agent (31–33). Nonetheless, normal human serum contained antigenic material reacting with rabbit anti-AHF, a result reported also by others (19, 20, 24). Presumably then, the inactivation of AHF does not result in the total destruction of its immunochemical character. Preliminary experiments suggest that the mobility of AHF may be altered by treatment with thrombin, but the genuineness of this observation is not yet certain.

A rare disorder, first described by Oeri, Matter, Isenschmid, Hauser, and Koller (34), is the congenital coexistence of deficiencies of AHF and proaccelerin. In one patient, normal amounts of AHF-like antigen were detected immunologically, despite a severe functional deficiency. Similarly, in a single patient with a high titer of circulating anticoagulant, first detected *post partum,* precipitating antigen was demonstrable. Antibody blocking studies could not be performed in this patient or in classic hemophiliacs with circulating anticoagulants, for these interfered with the assay procedure used.

A disease indistinguishable from human hemophilia has been described in dogs (35). Using antibody blocking tests, McLester and Wagner were unable to demonstrate antigenic material in hemophilic dogs. Whether this implies that hemophilic dogs do not synthesize AHF, or whether technical differences account for this result, is not yet clear.

Finally, additional evidence for the specificity of the antibody described here comes from a study of hemophilic carriers to be reported separately (36). As might be predicted from the data obtained in patients with classic hemophilia, proved carriers have, on the average, about one-half as much functional AHF as AHF-like antigenic material. The procedures used allowed the identification of a much higher proportion of hemophilic carriers than in our previous experience.

ACKNOWLEDGMENTS

Mrs. James Christie, Mrs. Mary Quinn Zyrkowski, and Miss Catherine Lynch provided invaluable technical help. Dr. Arthur S. Littell aided in the statistical analysis of our data.

This study was supported in part by grant HE 01661 from the National Heart and Lung Institute, the National Institutes of Health, U. S. Public Health Service, and by grants from the American Heart Association, and in part by United States Army Medical Research and Development Command Contract No. DADA-C-9006.

REFERENCES

1. Johnson, A. J., J. Newman, M. B. Howell, and S. Puszkin. 1967. Purification of antihemophilic factor (AHF) for clinical and experimental use. *Thromb. Diath. Haemorrh. Suppl.* **26**: 377.
2. Ratnoff, O. D., L. Kass, and P. D. Lang. 1969. Studies on the purification of antihemophilic factor (Factor VIII) II. Separation of partially purified antihemophilic factor by gel filtration of plasma. *J. Clin. Invest.* **48**: 957.
3. Laurell, C. 1966. Quantitiative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* **15**: 45.
4. Ratnoff, O. D., R. E. Botti, R. T. Breckenridge, and A. S. Littell. 1964. Some problems in the measurement of antihemophilic activity. *In* The Hemophilias, International Conference on Hemophelia, Washington, D. C., 1963. K. M. Brinkhous, editor. University of North Carolina Press, Chapel Hill, N. C. 3.
5. Prentice, C. R. M., R. T. Breckenridge, and O. D. Ratnoff. 1967. Studies on the conversion of prothrombin to thrombin: with notes on the cation requirement for this reaction. *J. Lab. Clin. Med.* **69**: 229.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265.
7. Seibert, R. H., A. Margolius, Jr., and O. D. Ratnoff. 1968. Observation on hemophilia, parahemophilia, and coexistent hemophilia and parahemophilia. *J. Lab. Clin. Med.* **52**: 449.
8. Tocantins, L. M. 1954. Hemophilic syndromes and hemophilia. *Blood.* **9**: 281.
9. Biggs, R., and J. M. Matthews. 1963. The treatment of haemorrhage in von Willebrand's disease and the blood level of Factor VIII (AHG). *Brit. J. Haematol.* **9**: 203.
10. Cornu, P., M. J. Larrieu, J. Caen, and J. Bernard. 1963. Transfusion studies in von Willebrand's disease: effect on bleeding time and factor VIII. *Brit. J. Haematol.* **9**: 189.
11. Nilsson, I. M., M. Blombäck, and B. Blombäck. 1959. Von Willebrand's disease in Sweden. Its pathogenesis and treatment. *Acta Med. Scand.* **164**: 263.
12. Krivit, W., R. J. Desnick, C. A. Mapes, and C. C. Sweeley. 1970. Recent advances in Fabry's disease. *Trans. Ass. Amer. Physicians.* In press.
13. Hershgold, E., L. Silverman, A. Davison, and M. Janszen. 1967. Native and purified Factor VIII: molecular and electron microscopical properties and a comparison of hemophilic plasma. *Fed. Proc.* **26**: 488.
14. Barrow, E. M., and J. B. Graham. 1969. The dissociation of plasma antihemophilic factor by succinic anhydride. *Fed. Proc.* **28**: 746.
15. Richards, M. D., and T. H. Spaet. 1956. Immunization of rabbits against human antihemophilic factor. *Blood.* **11**: 473.
16. Shanberge, J. N., and I. Gore. 1957. Studies on the immunologic and physiologic activities of antihemophilic factor (AHF). *J. Lab. Clin. Med.* **50**: 954.
17. Berglund, G. 1962. Studies of the inhibitory activity of specific antisera to some clotting factors in human plasma. *Brit. J. Haematol.* **8**: 204.
18. Adelson, E., J. J. Rheingold, O. Parker, M. Steiner, and J. C. Kirby. 1963. The survival of factor VIII (antihemophilic globulin) and factor IX (plasma throm-

boplastin component) in normal humans. *J. Clin. Invest.* **42**: 1040.
19. Piper, W., and M. H. Schreier. 1964. Über den immunologischen Nachweis von Faktor VIII-Protein im Bluterplasma und seine Bedeutung für das Verstandnis der Haemophilie A. *Thromb. Diath. Haemorrh.* **11**: 423.
20. Uszynski, L. 1966. The immunological properties of factor VIII. I. Studies on the inhibitory activity of specific antisera to human antihemophilic globulin. *Thromb. Diath. Haemorrh.* **16**: 559.
21. Bennett, E. 1970. Immunological studies in haemophilia. *Clin. Sci.* **38**: 118.
22. Uszynski, L. 1967. The immunological properties of factor VIII. II. The immunoelectrophoretic studies. *Thromb. Diath. Haemorrh.* **18**: 325.
23. Vainer, H., and J. Caen. 1965. Étude immuno-électrophorétique des plasmas d'Hemophile A (avec et sans anticoagulant circulant) et de maladie de Willebrand. *Nouv. Rev. Franc. Hémat.* **4**: 691.
24. Goudemand, M., M. Foucaut, A. Hutin, and A. Parquet-Gernez. 1963. Les anticoagulants circulants antifacteur VIII au cours de l'hémophilie A (nature et mécanisme d'action). *Nouv. Rev. Franc. Hémat.* **3**: 703.
25. Abildgaard, C. F., J. Vanderheiden, A. Lindley, and F. Rickles. 1967. Studies of cross-reactivity of acquired factor VIII inhibitor activity in hemophilic plasma. *Thromb. Diath. Haemorrh.* **18**: 354.
26. Hoyer, L. W., and R. T. Breckenridge, 1968. Immunologic studies of antihemophilic factor (AHF, Factor VIII): cross-reacting material in a genetic variant of hemophilia A. *Blood.* **32**: 962.
27. Denson, K. W. E., R. Biggs, M. D. Haddon, R. Borrett, and K. Cobb. 1969. Two types of haemophilia (A^+ and A^-): a study of 48 cases. *Brit. J. Haematol.* **17**: 163.
28. Feinstein, D., M. N. Y. Chong, C. K. Kasper, and S. I. Rapaport. 1969. Hemophilia A: polymorphism detectable by a factor VIII antibody. *Science (Washington).* **163**: 1071.
29. Spaet, T. H., and B. G. Kinsell. 1954. A circulating anticoagulant selectively active against human antihemophilic factor (autoimmune hemophilia). *Stanford Med. Bull.* **12**: 246.
30. Penick, G. D. 1957. Some factors that influence utilization of antihemophilic activity during clotting. *Proc. Soc. Exp. Biol. Med.* **96**: 277.
31. Quick, A. J., C. V. Hussey, and E. Epstein. 1953. Activation of thromboplastinogen by thrombin. *Amer. J. Physiol.* **174**: 123.
32. Therriault, D. G., J. L. Gray, and H. Jensen. 1957. Influence of thrombin on rate of prothrombin conversion. *Proc. Soc. Exp. Biol. Med.* **95**: 207.
33. Rapaport, S. I., S. Schiffman, M. J. Patch, and S. B. Ames. 1963. The importance of activation of antihemophilic globulin and proaccelerin by traces of thrombin in the generation of intrinsic prothrombinase activity. *Blood.* **21**: 221.
34. Oeri, J., M. Matter, H. Isenschmid, F. Hauser, and F. Koller. 1954. Angeborener Mangel an Faktor V (Parahaemophilie) verbunden mit echter Haemophilie A bei zwei Brüdern. *Ann. Paediat.* 1 (Suppl. 58): 575.
35. McLester, W. D., and R. H. Wagner. 1965. Antibody to antihemophilic factor and its lack of reaction with hemophilic plasma. *Amer. J. Physiol.* **208**: 499.
36. Zimmerman, T. S., O. D. Ratnoff, and A. S. Littell. 1971. Detection of carriers of classic hemophilia using an immunologic assay for antihemophilic factor (factor VIII). *J. Clin. Invest.* **50**: 000.
37. Snedecor, G. W. 1956. Statistical Methods, Applied to Experiments in Agriculture and Biology. Ames, Iowa State College Press, Iowa. 5th edition. 251.