

Factor-VIII-related antigen: Multiple molecular forms in human plasma

(crossed immunoelectrophoresis/liver disease/disseminated intravascular coagulation/von Willebrand's Factor/
chromatographic separation)

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ABSTRACT Analysis of Factor-VIII-related antigen (vW_{Ag}) in normal plasma by crossed immunoelectrophoresis reveals an asymmetric pattern indicative of molecular heterogeneity. This asymmetric pattern apparently results from the presence of multiple molecular forms of vW_{Ag} with distinct, though partially overlapping, mobilities on crossed immunoelectrophoresis. Evidence for this conclusion has been obtained by separating these forms, one from another, utilizing cryoprecipitation, agarose gel chromatography, and ion-exchange chromatography.

Variation in the relative distribution of vW_{Ag} forms in liver disease and disseminated intravascular coagulation suggests that the processes which govern generation and/or breakdown of these forms can be altered by disease.

Molecules expressing Factor-VIII-related antigen (von Willebrand's antigen, vW_{Ag}) are decreased in the plasmas of individuals with typical von Willebrand's disease though present in normal amounts in plasmas from individuals with hemophilia A. The concentration of plasma vW_{Ag} in normal plasmas correlates well with plasma Factor VIII procoagulant activity ($VIII_c$) and with the activity of a plasma factor required for platelet adhesiveness and ristocetin-induced platelet aggregation (von Willebrand's Factor, vW_F) (1, 2). Both of these activities copurify with vW_{Ag} under a variety of conditions, suggesting that they reside on the vW_{Ag} molecule (3, 4). However, additional studies have suggested antigenic differences between molecules responsible for procoagulant activity and those expressing vW_{Ag} (5). In addition, the two can be separated to various degrees by agarose gel chromatography at high strength (6).

We now report that molecules expressing vW_{Ag} are present in normal human plasma in several different forms when analyzed by crossed immunoelectrophoresis. These forms can be separated one from another by cryoprecipitation, agarose gel chromatography at *physiologic* salt concentrations, and anion exchange chromatography. The relative distribution of vW_{Ag} in plasmas from individuals with liver disease and disseminated intravascular coagulation often differs from that seen in normal individuals, indicating that as yet unidentified *in vivo* events influence the generation of different vW_{Ag} forms.

MATERIALS AND METHODS

Blood drawn with plastic syringes was routinely collected into citrate-phosphate-dextrose anticoagulant containing sufficient phenylmethylsulfonyl fluoride (PMSF) and Trasylol (Aprotinin, FBA Pharmaceuticals) to give a final concentration of 1 mM and 10 kallikrein inactivator units/ml, re-

spectively. In some experiments, benzamidine, 0.02 M, instead of PMSF, or ϵ -aminocaproic acid, 0.2 M, instead of Trasylol was used with similar results. Platelet-poor plasma was prepared by centrifugation of blood at $1500 \times g$ for 15 min followed by repeat centrifugation at $65,000 \times g$ for 45 min. Plasma for preparation of cryoprecipitate was snap-frozen with liquid nitrogen and stored at -70° . Cryoprecipitate was prepared by allowing the frozen plasma to thaw at 0° in an ice water-bath. The precipitate was collected by centrifugation at $27,000 \times g$ for 20 min. Cryoprecipitate from 50 to 100 ml of plasma was dissolved in 2 ml of barbital saline buffer (0.01 M barbital sodium, 0.015 M barbital, 0.125 M sodium chloride, pH 7.5).

Anion exchange chromatography of whole plasma or unconcentrated cryosupernatant plasma was performed in the following manner. Plasmas were treated with 1 mM DFP (diisopropylfluorophosphate) and then absorbed with aluminum hydroxide (Rehsorptar, Armour Pharmaceuticals, Kankakee, Ill.). One part of aluminum hydroxide was mixed with 10 parts of plasma and incubated with gentle stirring for 15 min at $22-25^\circ$. The aluminum hydroxide was removed by centrifugation at $17,000 \times g$ for 10 min. The plasma (100-400 ml) was then adjusted to a conductance between 1.5 and 6.0 mmho (mS), depending on the batch of DEAE-agarose (Bio-Rad) to be used, by dialysis against water using a Bio-Fiber device. Additional PMSF and Trasylol were added to a concentration of 1 mM and 10 units/ml, respectively; and the plasma was again centrifuged at $65,000 \times g$ for 45 min. Plasma was then applied to a column of DEAE-agarose which had been equilibrated with 0.01 M Tris, pH 7.5 buffer (Tris buffer) adjusted to a conductance of between 1.5 mmho and 6.0 mmho with sodium chloride and containing protease inhibitors (1 mM PMSF and/or 0.02 M benzamidine and Trasylol 10-100 units/ml or EDTA, 0.2 M). The column was washed with starting buffer and vW_{Ag} was then eluted with a linear sodium chloride gradient containing protease inhibitors.

Agarose gel chromatography was performed on 2.5×90 cm columns of Bio-Gel A-15m (Bio-Rad) which had been equilibrated with 0.15 M sodium chloride, 0.05 M Tris, pH 7.4 buffer containing 1 mM PMSF and 10 units/ml of Trasylol. The column was calibrated with blue dextran and the initial blue dextran peak was designated as the void volume (V_0).

Fractions were assayed for vW_{Ag} by quantitative immunoelectrophoresis (7). When necessary for crossed immunoelectrophoretic analysis, fractions were concentrated in an Amicon pressure apparatus at 10 pounds/inch² (69 kPa) using an XM 50 membrane.

Crossed immunoelectrophoresis was performed on 7.7×5.1 cm slides. Twenty microliter samples were placed in 4

Abbreviations: vW_{Ag} von Willebrand's antigen, Factor-VIII-related antigen; PMSF, phenylmethylsulfonyl fluoride.

mm diameter wells. Column samples were usually assayed in the eluting buffer. Dialysis of samples from DEAE-agarose chromatography into the higher ionic strength buffer used for agarose gel chromatography did not appreciably effect the results. Evan's blue dye was added to plasma samples, or Evan's blue dye mixed with 20% bovine serum albumin was added to column samples, as a marker. The first dimension (7.7 cm) was run in 0.9% weight/volume agarose (Van Waters and Rogers Scientific) in 0.025 M barbital buffer, pH 9.5, poured to a thickness of 1.5 mm, at a constant current of 3.75 mA per slide. Electrophoresis was continued until the marker dye migrated 6.5 cm (approximately 3 hr). Electrophoresis in the second dimension (5.1 cm) was carried out at a constant current of 5 mA per slide for 16 hr. Antiserum to vW_{Ag} was prepared as described previously (7). It precipitated with vW_{Ag} and inactivated Factor VIII procoagulant activity and von Willebrand Factor activity. An ammonium-sulfate-precipitated globulin fraction of the antiserum was used at a final concentration of 0.06–0.08 mg/ml (7). Slides were washed, dried, and stained with Coomassie blue as previously described (7).

RESULTS

Analysis of normal plasma vW_{Ag} by crossed immunoelectrophoresis reveals a characteristic asymmetric pattern (Figs. 1 and 2) indicative of heterogeneity. The following experiments demonstrate that this asymmetric pattern is due to the presence of multiple molecular forms of vW_{Ag} , with different mobilities on electrophoresis in agarose gel. First, cryoprecipitation was found to precipitate predominantly slowly moving forms of vW_{Ag} , leaving faster moving forms in the cryosupernatant plasma (Fig. 1, slides 5, 6, and 7). Second, 4% agarose gel chromatography of cryoprecipitated vW_{Ag} resolved it into multiple forms. Though vW_{Ag} was present in the void volume it also eluted in later fractions. When fractions from 1.0 to 2.0 V_0 were analyzed by crossed immunoelectrophoresis it was found that at least four different forms had been separated to various degrees one from the other (Fig. 2). Evidence that these forms were not in equilibrium one with another but did represent stable and distinct entities was obtained by mixing the form eluting at 1.04 V_0 with that eluting at 1.2 V_0 and analyzing the mixture by crossed immunoelectrophoresis. Two peaks are seen, each with the approximate mobility of the individual components analyzed separately (Fig. 2A, Slide 7). Though the precise position of different forms varied somewhat in five different column runs, utilizing five different cryoprecipitates, in each case those forms with faster mobility on crossed immunoelectrophoresis eluted after those forms with slower mobility.

Ion exchange chromatography on DEAE-agarose of cryosupernatant plasma allowed partial resolution of two fast-moving forms of vW_{Ag} (see Fig. 1, Slide 8), though on occasion only the fastest moving was present. These forms were further resolved by agarose gel chromatography. In a typical experiment, one form eluted at 1.2 V_0 and the second at 1.82 V_0 (see Fig. 2). The slower moving of the two forms was also present in most cryoprecipitates, the faster usually not. Thus a total of at least five different forms of vW_{Ag} were identified.

Third, evidence that the different vW_{Ag} forms exist in plasma and are not simply artifacts of the cryoprecipitation process was provided by chromatographic analysis of whole plasma on DEAE-agarose. Forms with faster mobility on

crossed immunoelectrophoresis eluted at a lower conductance and those with slower mobility eluted later (Fig. 2B).

The effect of blood clotting on vW_{Ag} forms was explored using blood drawn into either citrate, PMSF and Trasylol, or Trasylol alone. Both samples were incubated for 2 hr at 37°. vW_{Ag} in plasma containing citrate, PMSF, and Trasylol was then compared with that in serum containing only Trasylol by crossed immunoelectrophoretic analysis. A moderate increase in the faster moving forms was observed (see Fig. 1) in serum.

Plasma obtained from blood drawn into a plastic syringe containing heparin (final concentration, 10 units/ml) as well as PMSF and Trasylol, or citrate and the platelet cathepsin inhibitor *N*-carbobenzoxy- α -L-glutamyl-L-tyrosine (0.5 mM final concentration), showed a pattern similar to plasma from blood drawn into a plastic syringe and transferred to a plastic tube containing citrate anticoagulant with or without PMSF and Trasylol. However, when vW_{Ag} was isolated from plasma by column chromatography in the *absence* of protease inhibitors, relative increases in the faster-moving vW_{Ag} forms were sometimes seen.

Individual plasma samples showed the same pattern on repeated crossed immunoelectrophoretic analysis and different plasma samples from the same normal individual showed little or no variation from day to day. Samples of plasma vW_{Ag} from 10 cases of liver disease and two cases of disseminated intravascular coagulation were compared with plasmas from normal individuals. Increases in total vW_{Ag} were observed in most of the plasmas from these two disease states. The forms with intermediate or faster mobility were usually increased to a greater degree than forms with slower mobility (Fig. 1).

vW_{Ag} in plasmas from five individuals with hemophilia A were analyzed by crossed immunoelectrophoresis. Four had Factor VIII procoagulant activities of less than 1% and one had an activity of 5%. The patterns obtained were similar to those of normal plasmas (Fig. 1, Slide 5).

DISCUSSION

These studies demonstrate that vW_{Ag} exists in normal human plasma not as a single species, but rather as at least five distinct forms with different mobilities on crossed immunoelectrophoresis. The differing behavior of these forms under a variety of physical conditions further distinguishes them one from another. The slow mobility forms are separated from those with rapid mobility by cryoprecipitation, the latter remaining largely in the supernatant. Those with faster mobility elute from DEAE-agarose columns at lower conductance than the slower forms, suggesting that charge differences may exist between them and may account in part for the observed differences in behavior on crossed immunoelectrophoresis. Size differences are suggested by the behavior of vW_{Ag} forms on agarose chromatography. The slowest forms are recovered in, or just after, the void volume, whereas the fastest forms elute considerably later. Variable interaction of different forms with the agarose on a basis other than size may be responsible for this behavior, however (8). The existence of the vW_{Ag} forms as independent molecular entities is supported by the absence of equilibrium redistribution to a symmetrical pattern following mixing of forms which elute at different positions from the columns. This ability to clearly resolve forms of differing mobilities indicates that the asymmetric pattern seen in whole plasma is caused by the presence of a number of sets of vW_{Ag} molecules with overlapping mobilities.

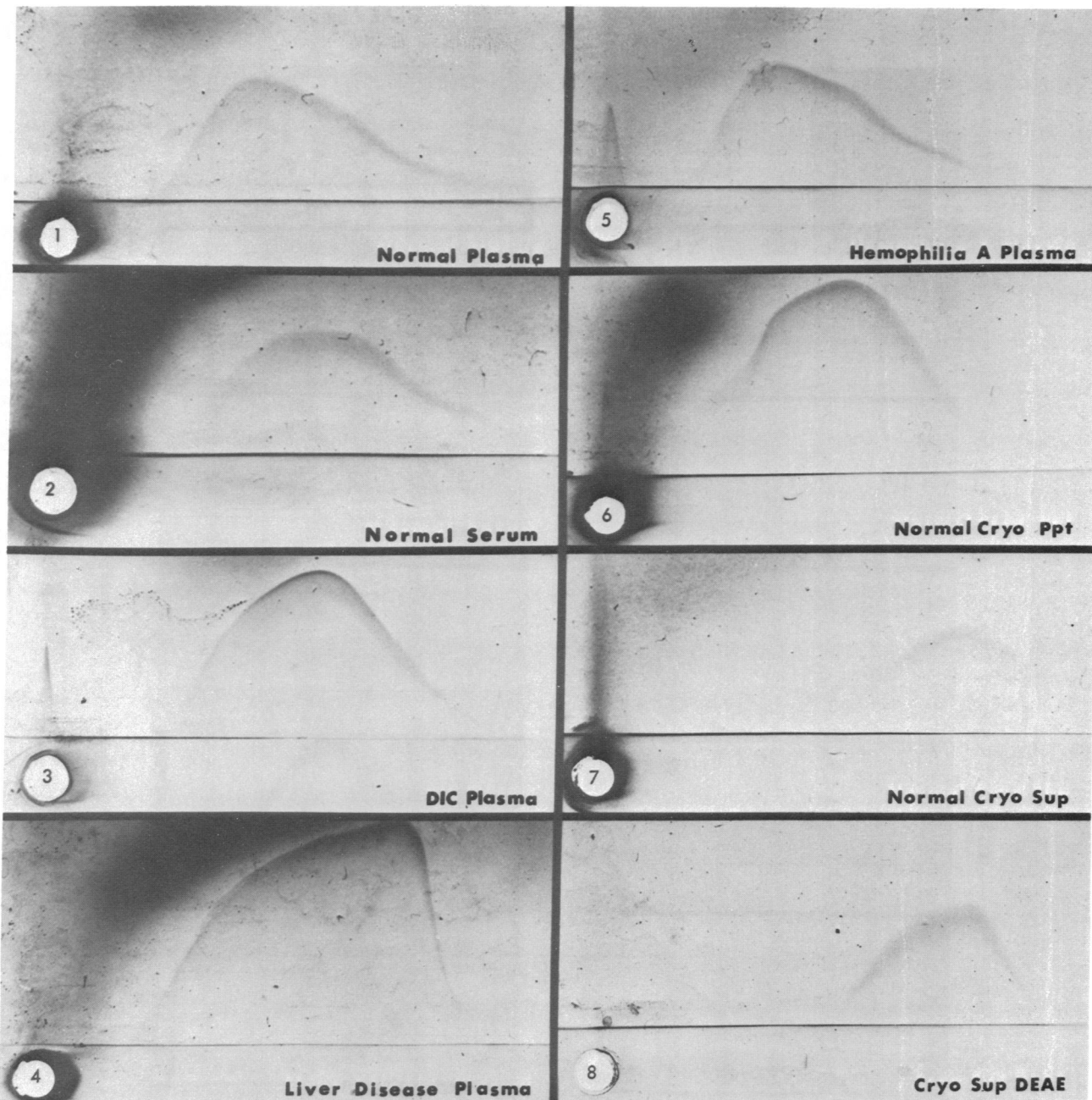


FIG. 1. Crossed immunoelectrophoretic analysis of vW_{Ag} in plasma, serum, and plasma fractions. The anode is to the right in the first dimension and at the top in the second dimension. As a marker Evan's blue dye or Evan's blue dye and bovine serum albumin were mixed with the samples and electrophoresed 6.5 cm in the first dimension.

Slide 1. Typical normal plasma. Marked asymmetry, indicative of heterogeneity, is evident. Compare with Fig. 2A, Slides 2–6 in which five vW_{Ag} forms with differing mobilities in agarose gel electrophoresis have been separated from one another and give symmetrical patterns. *Slide 2.* Typical normal serum. Blood (from which plasma analyzed in Slide 1 was also obtained) was allowed to clot for 2 hr at 37° in the presence of Trasylol, 100 units/ml. A moderate shift to faster vW_{Ag} forms is seen. *Slide 3.* Plasma from an individual with disseminated intravascular coagulation. A marked increase in vW_{Ag} of intermediate mobility is seen. *Slide 4.* Plasma from an individual with liver disease. A marked increase in the forms of fast mobility is observed. In other cases of liver disease, an increase in forms with slower or intermediate mobility was also seen. *Slide 5.* Plasma from an individual with Hemophilia A. The pattern is essentially the same as that seen in normal individuals. Retention of vW_{Ag} at the origin occurred with a minority of normal and pathologic plasmas. *Slide 6.* Cryoprecipitate prepared from normal plasmas. Slower vW_{Ag} forms predominate. *Slide 7.* Supernatant plasma remaining after cryoprecipitation (cryosupernatant). The supernatant plasma was concentrated on a Bio-Fiber device prior to a crossed immunoelectrophoretic analysis. Only the faster moving forms are detectable. *Slide 8.* Cryosupernatant, absorbed to DEAE-agarose and eluted with a sodium chloride gradient. Two fast-moving forms are seen. These were further resolved by agarose gel chromatography (see Fig. 2, Slides 5 and 6).

The relationship of the different vW_{Ag} forms to Factor VIII procoagulant activity and von Willebrand's Factor activity is as yet unclear. Factor VIII procoagulant activity: vW_{Ag} ratios are often decreased in liver disease (9), and we have now demonstrated differences in the relative concen-

trations of different vW_{Ag} forms in some liver disease patients. It is possible that certain of the molecular forms may have reduced specific activities in respect to procoagulant activity and/or von Willebrand's Factor activity. Increases in such forms may account for the relative increase in vW_{Ag}

Panel A - Agarose Gel Chromatography

Panel B - DEAE Agarose Chromatography

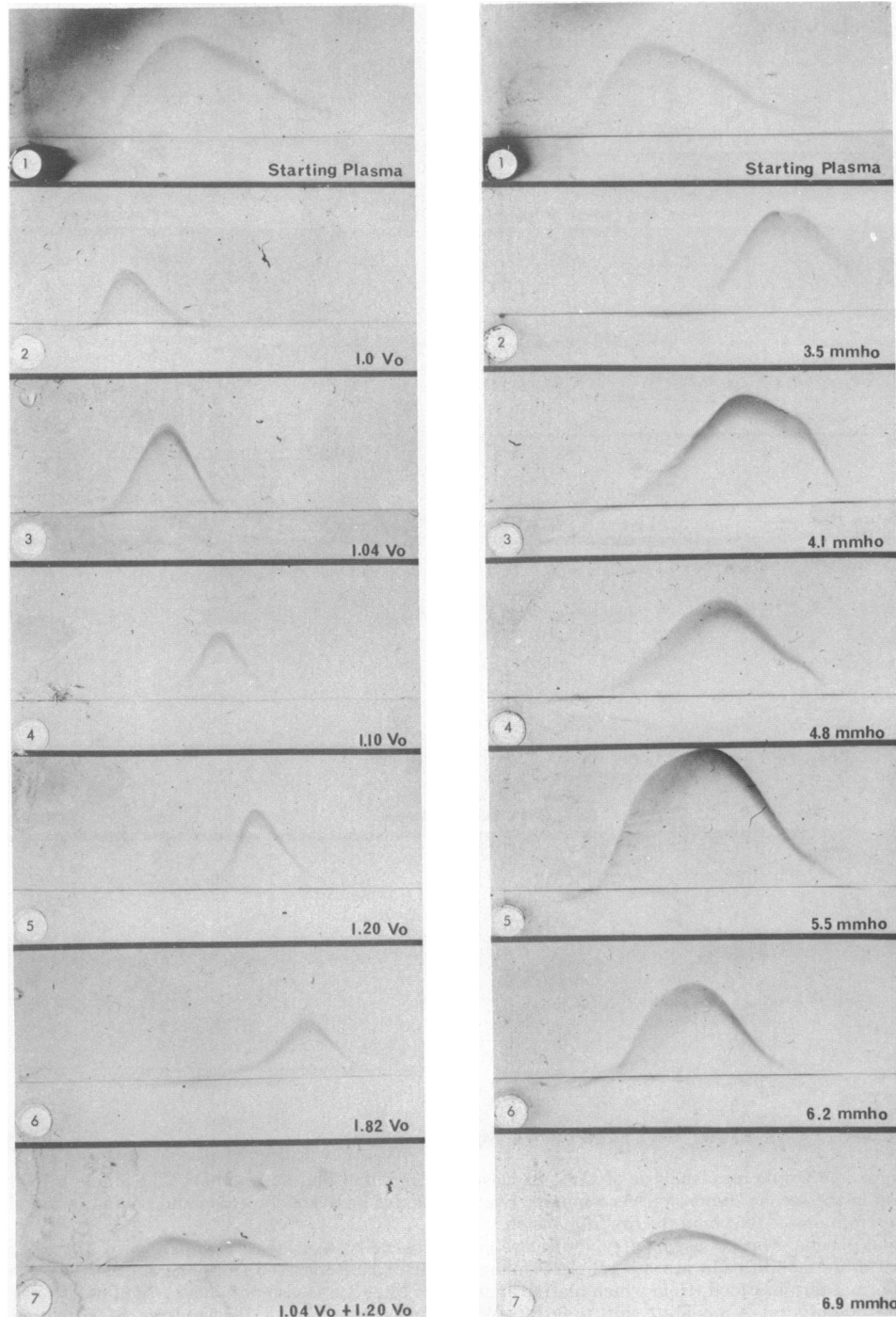


FIG. 2. Panel A. Resolution of vW_{Ag} forms by agarose gel chromatography. Cryoprecipitate (Slides 2-4), or cryosupernatant which had been chromatographed on DEAE-agarose (Slides 5 and 6) was subjected to agarose gel chromatography. vW_{Ag} forms with slowest mobility on crossed immunoelectrophoresis eluted first with those of faster mobility eluting later. The form eluting at $1.2 V_0$ was usually present in both cryoprecipitate and cryosupernatant, whereas the slower-moving forms were present only in the cryoprecipitate. When vW_{Ag} forms eluting at different portions of the column were mixed together and analyzed by crossed immunoelectrophoresis, two peaks were seen with the approximate mobility of the individual components analyzed separately (Slide 7), indicating that these forms are not in equilibrium one with another, but represent stable and distinct entities.

Panel B. Partial resolution of vW_{Ag} forms by DEAE-agarose chromatography. Fresh whole plasma was applied to a DEAE-agarose column at a conductance of 1.5 mmho, pH 7.5, and eluted with a linear sodium chloride gradient. The vW_{Ag} forms with fastest mobility on crossed immunoelectrophoresis eluted at a lower conductance than those of slower mobility which eluted at a higher conductance.

as compared to Factor VIII procoagulant activity that occurs in liver disease. An increase in vW_{Ag} forms with low specific activities may result in misclassification of some women as

carriers of hemophilia A. It may also account for the relatively wide range of Factor VIII procoagulant activity: vW_{Ag} ratios observed in normal individuals, a factor which

limits reliable identification of carriers. The possibility exists that antisera to vW_{Ag} produced in different laboratories may vary in their interactions with the different vW_{Ag} forms. Such variations could contribute to the observed differences between laboratories in the detection of carriers.

The basis of multiple vW_{Ag} forms in plasma remains to be elucidated. The minimal change which occurs during blood coagulation *in vitro* suggests that the observed heterogeneity of vW_{Ag} is not the result of thrombin generation or activation of other enzymes during the interval between venipuncture and equilibration with the anticoagulant and protease inhibitors. *In vivo* proteolysis may be responsible, however. Alternative explanations include the possibility that the different forms may represent different polymeric combinations of a common subunit or of different subunits, or complex formation with other molecules.

Recently, variants of von Willebrand's disease have been described in which the vW_{Ag} is present but migrates with aberrantly rapid mobility on crossed immunoelectrophoretic analysis (10, 11) and/or elutes after the void volume on agarose gel chromatography (12). The findings in this study suggest that such forms may be present in low concentration in normal individuals. Elucidation of the molecular basis for the multiple forms of vW_{Ag} in normal individuals, their relative variation in various pathologic and physiologic states, as well as their relationship to the dysfunctional forms of vW_{Ag} present in some forms of von Willebrand's disease, represents an important challenge to our understanding of the biology of this molecule.

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