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Separation of Bovine Factor VIII-Related Antigen (Platelet Aggregating Factor) from Bovine Antihemophilic Factor[1] (38398)

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Work in our laboratory (1) as well as others (2, 3) supports the hypothesis that in human plasma, factor VIII-related antigen[2] and factor VIII are located on separate molecules. The demonstration that patients with classic hemophilia have normal levels of factor VIII-like antigen, while those with von Willebrand's disease have low levels (4, 5), leads to the speculation that the measurement of the factor VIII-like antigen is in fact a measurement of the von Willebrand's factor. Weiss *et al.* (6) defined the von Willebrand's factor as the factor in normal plasma that is required for the antibiotic, ristocetin, to aggregate washed, normal human platelets and developed an assay on that basis. Using these two measurements in conjunction, Weiss and Hoyer (3) have shown that the von Willebrand's factor remains with the factor VIII-like antigen (in the void volume) upon gel filtration chromatography in 0.8 *M* NaCl. Under these conditions factor VIII activity shifted to a lower molecular weight form. Bovine plasma has an additional factor, namely the human platelet aggregating factor (PAF)[3] (7–9). The present work relates the PAF in bovine plasma with the factor VIII-like antigen and von Willebrand's factor as defined by Weiss (6). Data are presented indicating that these three measurable properties are associated with the same molecular species and are separable from bovine factor VIII coagulant activity.

Materials and Methods. Bovine plasma preparations: Bovine blood was collected in 3.8% trisodium citrate (one part citrate to nine parts blood) during slaughter of adult animals. The plasma was adsorbed for 15 min at 10° with 1/20 part of 2% $Al(OH)_3$ gel (Reheis). Solid ammonium sulfate (Mallinckrodt) was added at 10° to 18% saturation (102 g/liter or 15% saturation (84 g/liter) followed by centrifugation at 2000*g* for 0.5 hr at 0°. Frequently a floating disc of precipitated protein was observed as well as a precipitate that sedimented. Both were removed, dissolved in 0.02 *M* Imidazole–HCl, 0.15 *M* NaCl, pH 6.6, and the remaining liquid frozen at −20°. Subsequent thawing until only 0.1 vol of ice remained was followed by a 0.5 hr spin at 2000*g* at −3°. The precipitate was called a cryoprecipitate.

Chromatography. A Sepharose 4B column (Pharmacia) (3.8 × 90 cm) was packed in Imidazole-buffered saline at a flow rate of 40 ml/hr. QAE Sephadex A-25 (Pharmacia) was swollen for 24 hr in buffer and a column (2.8 × 34 cm) packed in buffered saline. Fractions containing 4 ml were collected at room temperature.

Assay methods. Factor VIII was assayed by the partial thromboplastin time of Langdell *et al.* (10) with human hemophilic plasma as the substrate and kaolin as the activator. Antigen was measured by the quantitative electroimmunophoresis technique of Laurell (11) as modified by Zimmerman *et al.* (5). Units of activity ($VIII_{coag}$ or $VIII_{R-Ag}$) refer to the amount of the activity in one ml of adsorbed normal bovine plasma.

Platelet aggregating activity was measured on microtiter plates using either washed human or bovine platelets. Platelet washing was carried

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[2] Defined as that factor in plasma purified 1000-fold with respect to factor VIII coagulant activity which induces a precipitating rabbit antibody against the same or a similar preparation. This can be quantitated immunologically and is not necessarily related to factor VIII.

[3] Abbreviations—PAF, human platelet aggregating factor in bovine plasma, $VIII_{R-Ag}$, factor VIII-like antigen; $VIII_{coag}$, procoagulant activity associated with factor VIII; vWf, von Willebrand's factor.

out three times using an albumin cushion as described by Walsh (12). The PAF titer was recorded as the highest dilution which caused detectable platelet aggregation after 2 min of shaking. The aggregation system consisted of 10 μl of washed platelets which were added to 10 μl of test solution.

Results. Antibody inhibition. Rabbit antiserum to bovine factor VIII (20 μl, 0.35 mg) was incubated with citrated bovine plasma (100 μl) for 30 min at 25°, and then 10 μl aliquots taken for measurement of $VIII_{coag}$ and PAF titer. Two controls were run using either saline or normal rabbit serum in the same manner. Complete inhibition of PAF (>98%) took place while the partial thromboplastin time was inhibited by only 39% (61–67 sec). Control plasma gave a PAF titer of 1/80.

Ammonium sulfate precipitation. Table I shows how a typical 18% ammonium sulfate fractionation enriches PAF[2] and $VIII_{R-Ag}$ relative to $VIII_{coag}$ with respect to normal plasma. Subsequent cryoprecipitation of the supernate, yielded a fraction enriched in $VIII_{coag}$ to antigen and PAF by fivefold over normal plasma.

Chromatographic separation. In a separate fractionation, from 2.5 liter of $Al(OH)_3$ adsorbed bovine plasma, a 15% saturation of ammonium sulfate was made. The resulting floating disc of protein was removed after centrifugation and taken up in 16 ml of Imidazole buffered saline (32 units/ml $VIII_{coag}$). Unlike cryoprecipitates, it maintained complete $VIII_{coag}$ activity in the cold for several days. Application to a Sepharose 4B column yielded 240 units of $VIII_{coag}$ (300 units $VIII_{R-Ag}$) in the void volume, with no detectable fibrinogen. A portion (180 units $VIII_{coag}$ in 60 ml) was applied directly to a QAE A-25 Sephadex column, Fig. 1. After all of the material had entered the column, a gradient starting from 0.15 to 0.6 *M* NaCl was begun. $VIII_{coag}$ consistently eluted at higher ionic strength than $VIII_{R-Ag}$. In fact $VIII_{coag}$ continued to leach off the column after 100 ml of 2.0 *M* NaCl had passed through; nevertheless the recovery of factor $VIII_{coag}$ was greater than 50%. Fractions 50 and 70 were examined in particular and Table II shows how these fractions compare with regard to PAF, $VIII_{coag}$ and bovine vWf (as defined by Weiss *et al.* (5)). The titer which will aggregate washed human platelets in the absence of ristocetin and washed bovine platelets in the presence of ristocetin appears to be about the same and correlates with high antigen values rather than high $VIII_{coag}$.

Platelet aggregation studies. Washed bovine platelets obtained by venipuncture of a calf did not aggregate with bovine plasma alone. Ristocetin added to a final concentration of 2 mg/ml caused the platelets to aggregate only in the presence of a bovine plasma fraction. Bovine serum or human plasma in conjunction with ristocetin would not cause the platelets to aggregate. From the titer indicated in Table II, this bovine vWf appears to be associated with the PAF and $VIII_{R-Ag}$.

Discussion. Observations that a single ammonium sulfate fraction (Table I) can enrich $VIII_{R-Ag}$ to $VIII_{coag}$ relative to that found in citrated bovine plasma, can be criticized using the argument that during the process of fractionation, $VIII_{coag}$ could have been preferentially destroyed by the salt (9). This does not seem likely particularly in noting the reverse ratio of $VIII_{coag}$ to $VIII_{R-Ag}$ after cryoprecipitation of supernate. The antibody data show a selective inhibition of platelet aggregation activity (98%) relative to $VIII_{coag}$ (39%). Thus either PAF is more antigenic, or more likely it is found in greater abundance in $VIII_{coag}$ concentrates. This does not rule out the possibility of two or more antigenic sites on the same molecule. However, chromatographic separation (Fig. 1) of $VIII_{R-Ag}$ (PAF) from $VIII_{coag}$ would seem to rule out the one molecule, two site hypothesis. The fact that the titer of the PAF and of the ristocetin co-factor

TABLE I. Ratio of PAF and Antigen to Factor VIII in Ammonium Sulfate Precipitation.

	Vol. (ml)	Units/ml $VIII_{coag}$	$VIII_{R-Ag}$	PAF titer	$VIII_{R-Ag}$/ $VIII_{coag}$
Adsorbed bovine plasma	3500	1.0	1.0	1/20	1
18% (disc)	150	6.4	16.0	1/320	2.5
18% (pellet)	60	1.3	3.5	1/80	2.7
Supernate	3500	0.28	—	—	—
Cryoppt.	60	5.0	1.0	1/10	0.2

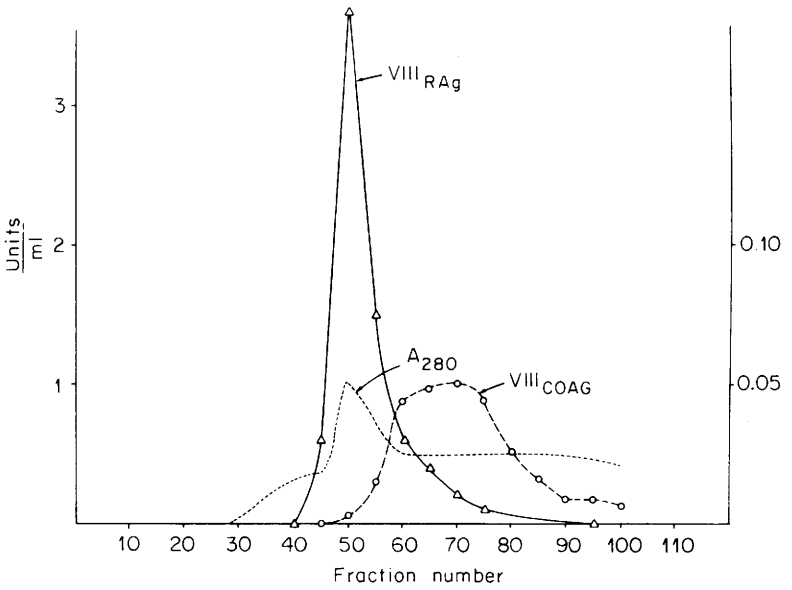

FIG. 1. Chromatography of QAE Sephadex A-25 (2.8 × 34 cm) of $VIII_{coag}$ (225 units $VIII_{R-Ag}$), 9 mg protein. Linear gradient from 0.15 to 0.6 *M* NaCl, in 0.02 *M* Imidazole–HCl buffer, pH 6.6.

activity (vWf) was higher at the peak $VIII_{R-Ag}$ fraction (#50) than at peak $VIII_{coag}$ activity (#70) (Table II), leads to the speculation that the $VIII_{R-Ag}$, PAF and vWf activities are all located on the same molecule. Caution however, should be taken in concluding this, as the mere observation that chromatographically they elute together is not criteria enough for identity.

Unlike the work of Griggs (9) who demonstrated separation of $VIII_{coag}$ from PAF and DEAE Sephadex; QAE Sephadex, a more strongly basic anion exchanger, removes essentially all of the $VIII_{coag}$ activity from PAF. Examination of the elution profile (Fig. 1) shows that most of the absorbance at 280 nm was associated with $VIII_{R-Ag}$ rather than $VIII_{coag}$. It now seems possible that bovine $VIII_{coag}$ is a protein present in such trace amounts that its physicochemical properties are not apparent in previously highly purified preparations (13). The properties described are likely those of the factor VIII-like antigen or PAF.

Summary. It was shown that in bovine plasma, factor VIII-related antigen can be separated from factor VIII coagulant activity by chromatography on QAE Sephadex. The suggestion is made that the factor in bovine plasma which aggregates human platelets and which aggregates washed bovine platelets in the presence of ristocetin is identical to the factor VIII-like antigen.

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TABLE II. Activities of Column Fractions 50 and 70 from Fig. 1.

Fraction	Units/ml $VIII_{R-Ag}$	Units/ml $VIII_{coag}$	$VIII_{R-Ag}$/ $VIII_{coag}$	Human platelet titer	Ristocetin bovine platelet titer
# 50	3.7	0.05	250	1/80	1/90
# 70	0.2	1.0	0.2	1/5	1/1

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