

## Some Factors Affecting Fibrinogen Precipitation by Ristocetin: Ultrastructure of Precipitates

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Fibrinogen in aqueous solution is precipitated by the antibiotic ristocetin. This reaction is inhibited by albumin and facilitated by low temperature. Resolubilized fibrinogen clots in the presence of thrombin. Ristocetin-precipitated fibrinogen takes the form of fibrils or clumps, composed of irregularly spaced, structureless particles. The addition of ristocetin to washed platelets suspended in fibrinogen-containing media produces fibrinogen clumps in both the media and in the surface cannicular system of the platelets. The changes in light transmission (aggregation curves) are due to both platelet aggregation and fibrinogen clumping. The

role of the latter is confirmed by the observation that the addition of ristocetin to inert latex particles suspended in fibrinogen solution produces typical aggregation curves. This phenomenon is prevented by the addition of albumin to the media. We conclude that (1) if fibrinogen is present in any artificial system, albumin should be included in the media to prevent fibrinogen precipitation; and (2) statements about aggregation of any particulated materials by ristocetin should not be based solely on light-transmission changes, but should also include a description of the morphologic appearance.

**R**ISTOCETIN CAUSES thrombocytopenia and hypofibrinogenemia when administered to human subjects.<sup>1</sup> It has been found that ristocetin produces aggregation of normal human and rabbit platelet-rich plasma,<sup>2</sup> but it has no effect upon samples of platelet-rich plasma obtained from patients with severe von Willebrand's disease (vWd).<sup>3</sup> Therefore, ristocetin may prove to be a useful tool in the diagnosis of this disorder.<sup>4-6</sup> Howard and Firkin<sup>3</sup> also noted that ristocetin causes precipitation of fibrinogen. Since fibrinogen concentrations in vWd patients are normal, the ristocetin-dependent platelet aggregation plasma factor (RAF) is unlikely to be fibrinogen; it is likely to be a factor lacking in vWd.<sup>7</sup> Although several publications attempting to elucidate the mechanism of ristocetin-induced platelet aggregation have appeared in the literature,<sup>8-9</sup> the relationship between ristocetin and fibrinogen remains relatively unexplored. For this reason, we have studied the interaction between these two substances. In this report we shall describe (1) several factors affecting this interaction, (2) the ultrastructure of ristocetin-precipitated fibrinogen, and (3) the relationship between this material and platelets.

### MATERIALS AND METHODS

Human albumin was purchased from Hyland Laboratories, Costa Mesa, Calif., as a 25% solution.

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Purified human fibrinogen was purchased from two sources: Cutter Laboratories, Berkeley, Calif., and Kabi Laboratories, Sweden. Both contain greater than 90% clottable protein.

Latex suspensions were obtained from Difco Laboratories, Detroit, Mich. (Bactolatex 0.81). The diameter of the latex particles was  $0.81 \mu$ . The preparation was diluted 1:100 in a buffered medium containing glucose, 1 mg/ml, 140 mM NaCl, 5mM KCl, 4mM sodium citrate, 3 mM citric acid, and 4 mg/ml fibrinogen. The pH of the medium was adjusted to 7.4 with Tris buffer. Portions of the medium also contained albumin, 5 mg/ml.

Ristocetin A was generously supplied by Mr. Grant Barlow of Abbott Laboratories, North Chicago, Ill. The material was dissolved in normal saline and stored at 4°C. Ristocetin stored in this manner was stable for at least 3 mo.

Bovine topical thrombin (Parke-Davis, Detroit, Mich.) was dissolved in saline and kept at -20°C.

### *Platelet Specimens*

Platelet-rich and platelet-poor plasmas (PRP and PPP) were prepared from venous blood drawn from laboratory personnel who had not taken aspirin or similar drugs in the preceding 2 wk. Blood was anticoagulated with buffered citrate (0.1 M), 1 part of anticoagulant to 9 parts of blood. Methods of preparation of PRP and PPP were previously described.<sup>10</sup> Platelets in all PRP and in some PPP specimens were counted by phase-contrast microscopy. Washed platelet suspensions (WPS) were prepared according to the technique of Rossi.<sup>11</sup>

### *Effect of Albumin on Ristocetin-induced Fibrinogen Precipitation*

Human fibrinogen was dissolved in Tris-saline buffer (Trizma base, 1.97 g; Trizma HCl, 5.32 g; NaCl, 8.5 g in 1 liter, pH 7.5, Sigma Chemicals, St. Louis, Mo.). An aliquot of fibrinogen was placed in a cuvette containing a stir bar. Human albumin, diluted with Tris-saline buffer, was added, and the cuvette was placed in a platelet aggregometer (Chrono-Log Corp., Broomal, Pa.) prewarmed to 37°C. Ristocetin was then introduced to the mixture, and the change in light transmission was recorded. Several studies were performed using the following albumin concentrations: 0, 45, 90, 180, 360, and 720 mg/100 ml. The concentrations of fibrinogen and ristocetin were held constant; they were 133 mg/100 ml and 1.33 mg/ml, respectively.

### *Effect of Temperature on Fibrinogen Precipitation and Platelet Aggregation by Ristocetin*

Citrated human plasma was placed either in an icebath or in a 37°C waterbath. Ristocetin at final concentrations of 0.5, 0.75, 1.0, 1.5, or 2.5 mg/ml was added to the plasma. The mixture was intermittently agitated, and it was examined for precipitates at 5 min. Fibrinogen precipitation was visually graded as 0, no precipitation; C, cloudy; F, filaments; or Cp, clumps. At the end of 5 min, phosphate-buffered glutaraldehyde, previously adjusted to the same temperature as the reaction mixture, was added to the tubes and the fixed material prepared for electron microscopy.<sup>10</sup> Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philip 300 electron microscope.

In other experiments, citrated PRP was exposed to 2.5 mg/ml of ristocetin at 0°C or 37°C, with or without constant stirring. After 5 min, the mixtures were fixed and examined by electron microscopy.

### *Effect of Ristocetin on the Ability of Thrombin to Clot Fibrinogen*

Ristocetin in final concentrations of 0, 0.5, 1.0, 1.5, 2.0, and 2.5 mg/ml was added to aliquots of citrated PPP. After incubation at 37°C for 3 min, the thrombin time of each sample of PPP was determined by adding 0.1 ml of thrombin (5 U/ml) to a cup containing 0.2 ml PPP-ristocetin mixture, and the clotting time was measured with a fibrometer (Bioquest, Cockeysville, Md.).

*Aggregometry and Ultrastructure of Washed Platelets and of Latex Particles Exposed to Ristocetin*

An aliquot of 0.2 ml of washed platelets or latex particles suspended in the medium containing fibrinogen (300 mg/ml), with or without albumin, was placed in a cuvette. After the addition of 0.01 ml of citrated PPP, the mixture was exposed to 0.02 ml of ristocetin (final concentration, 1.75 mg/ml), and the change in light transmission occurring over a 4-min period was recorded. The samples were then fixed for electron microscopy.

### RESULTS

When ristocetin was added to fibrinogen in aqueous solution, fibrinogen was readily precipitated, and the mixture immediately turned cloudy. This produced a decrease in light transmission. In samples in which the fibrinogen concentration was constant, the decrease in light transmission was directly related to the concentration of ristocetin. Albumin inhibited fibrinogen precipitation, as indicated by the prevention of the decrease of light transmission when ristocetin was added to the fibrinogen-albumin solution. The inhibitory effect

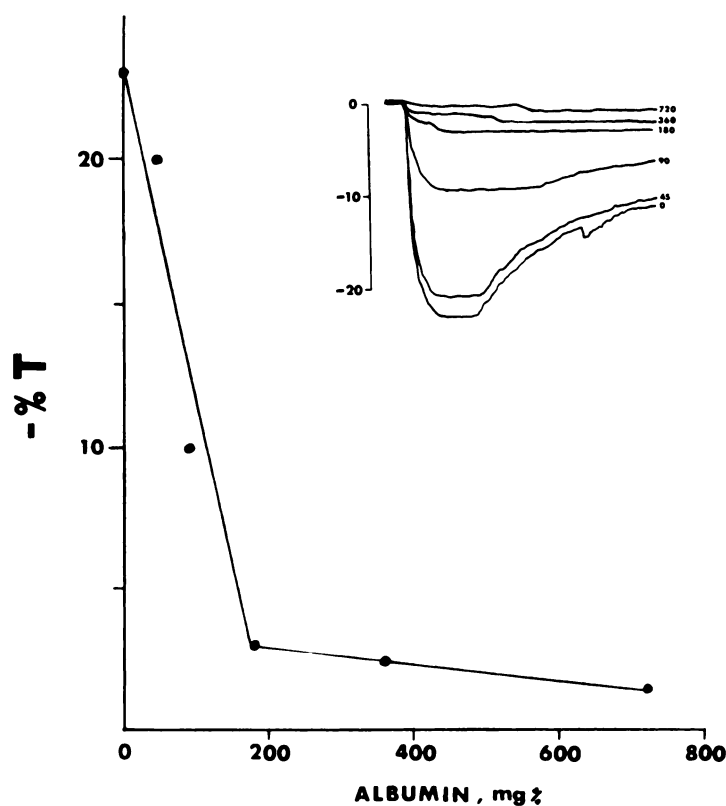


Fig. 1. Effect of albumin on the decrease of light transmission brought about by exposing fibrinogen solution to ristocetin. The initial increase in albumin concentration has a marked effect in preventing fibrinogen precipitation; further increases in albumin concentration have little additional effect. Inset is the actual recording. Concentrations of fibrinogen and ristocetin, 133 mg/100 ml and 1.33 mg/ml, respectively.

**Table 1. Effect of Temperature on Fibrinogen Precipitation by Ristocetin in Citrated Human PPP**

	Final Concentrations of Ristocetin (mg/ml)					
	0.5	0.75	1.0	1.5	2.0	2.5
0°C	C	C	F	F	Cp	Cp
37°C	0	0	0	0-C	C-Cp	F-Cp

0, no precipitation; C, cloudy; F, fibrils; Cp, clumps.

of albumin on ristocetin-induced precipitation of fibrinogen was proportional to the albumin concentration (Fig. 1). When the fibrinogen concentration was 133 mg/100 ml, precipitation by 1.33 mg/ml of ristocetin was prevented by 180 mg/100 ml of albumin. The protective effect of albumin on fibrinogen precipitation was limited at higher concentrations of ristocetin. For example, when the ristocetin concentration was increased to 2.5 mg/ml, 1000 mg/100 ml of albumin failed to prevent fibrinogen precipitation.

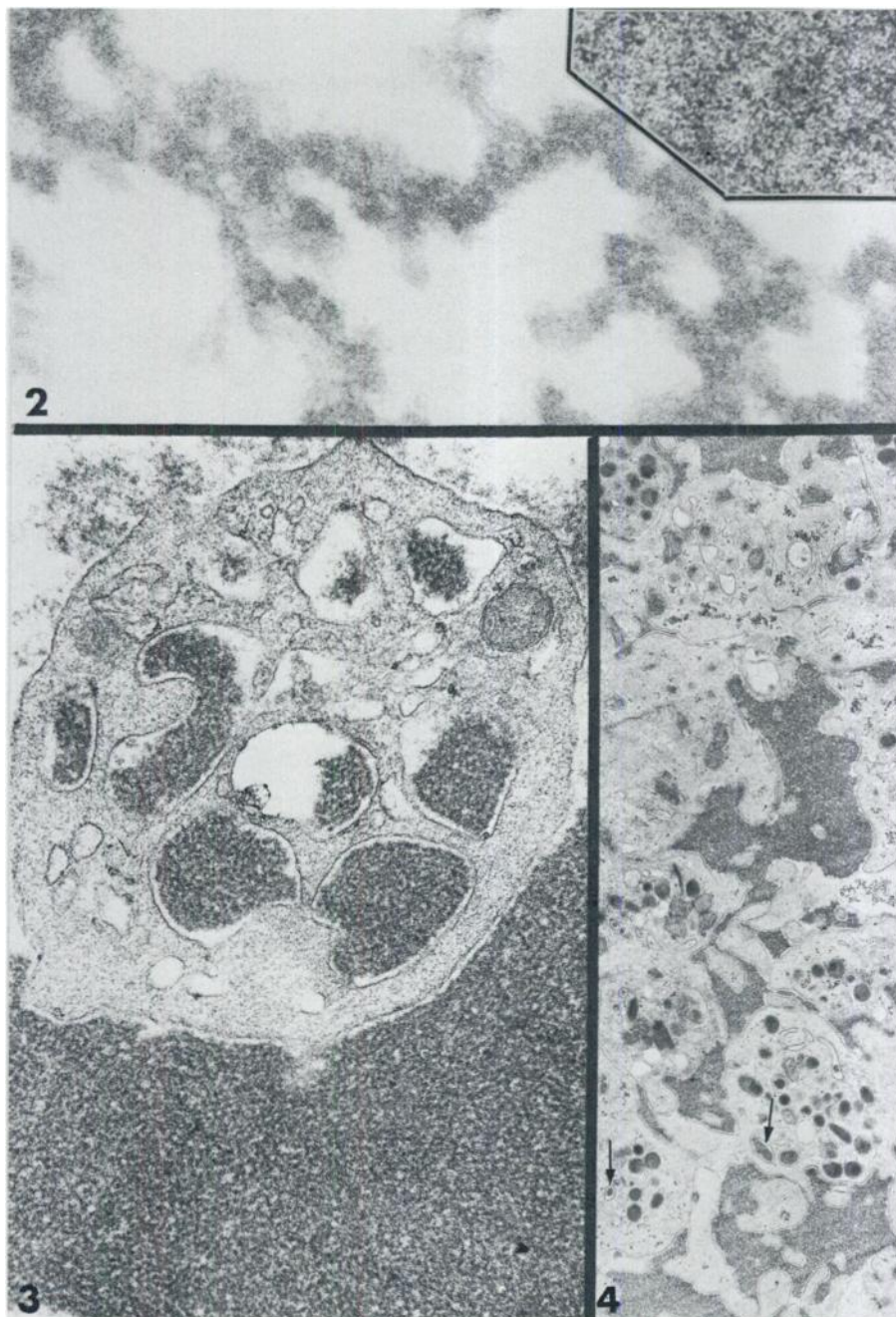
In constantly stirred, citrated PPP, ristocetin at a final concentration of 1.0 mg/ml caused no precipitation at 37°C. Higher concentrations of ristocetin, 2.5 and sometimes 2.0 mg/ml, did produce precipitation. Precipitation of fibrinogen by ristocetin was facilitated by lowering the temperature of the plasma or aqueous fibrinogen solution. The effect of temperature on fibrinogen precipitation in normal citrated human plasma as observed by the naked eye and/or by light microscopy is shown in Table 1. Fibrinogen precipitated at 0°C by low concentrations of ristocetin (1.0 or 1.5 mg/ml) could be redissolved at 37°C. Stirring or agitation expedited the solubilization. Low concentrations of ristocetin (0.5–1.5 mg/ml) shortened the thrombin time of normal plasma by 2–3 sec. High concentrations of ristocetin (2.5 mg/ml), however, prolonged the thrombin time (Table 2).

#### *Ultrastructure of Ristocetin-precipitated Fibrinogen: Presence of Fibrinogen Clumps in Platelet Surface Cannalicular System (SCS)*

Fibrinogen precipitated by ristocetin appeared as either filaments or as clumps, depending on the concentration of the antibiotic and the experimental conditions (see Table 1). The ultrastructure of the filamentous fibrinogen precipitates is shown in Fig. 2. The filaments were composed of irregularly spaced, fine granular particles of various size. The inset of the figure illustrates the precipitated fibrinogen at a higher magnification. There was no structural difference between fibrinogen precipitated from aqueous solution or from citrated plasma. In the presence of platelets, fibrinogen clumps were found in the platelet SCS (Fig. 3).

**Table 2. Effect of Ristocetin on Thrombin Time of Normal Citrated Plasma**

Ristocetin (mg/ml)	Thrombin Time (sec)
0	14.4
0.5	11.9
1.0	11.4
1.5	11.6
2.0	12.9
2.5	18.9



**Fig. 2.** Electron-microscopic appearance of ristocetin-precipitated fibrinogen. The precipitated material is filamentous and resembles fibrin strands under light microscopy. When viewed through the electron microscope, the material appears as chains of uneven thickness and electron density. At higher magnification (inset), the material is composed of fine particulate matter of various sizes.  $\times 6800$ ; inset,  $\times 430,900$ .

**Fig. 3.** Presence of ristocetin-precipitated fibrinogen in the surface canalicular system (SCS) of a platelet and outside a platelet. Ristocetin (1 mg/ml) was added to a PPP sample at  $0^{\circ}\text{C}$ .  $\times 38,500$ .

**Fig. 4.** Platelet clumping and fibrinogen precipitation in a PRP sample treated with 2.5 mg/ml ristocetin. The sample was constantly stirred at  $37^{\circ}\text{C}$ . Fibrinogen precipitates appear among clumped platelets as well as in platelet SCS (arrows).  $\times 8500$ .

Platelet aggregation and fibrinogen precipitation induced by ristocetin (2.5 mg/ml) were examined in stirred and unstirred samples of PRP at 0°C and at 37°C. Fibrinogen precipitation occurred after the addition of ristocetin regardless of the experimental conditions employed. However, platelet aggregation was observed only in stirred samples of PRP at 37°C (Fig. 4).

*Light-transmission Changes and Morphologic Alterations in Platelet and Latex Suspensions Exposed to Ristocetin: Effect of Albumin*

When platelet suspensions containing fibrinogen were exposed to ristocetin, there was a prompt decline in light transmission, but no subsequent increase occurred despite prolonged incubation. However, the addition of only 0.01 ml of plasma gave a biphasic curve, with an initial decrease in transmission followed by a progressive rise (curve 1, Fig. 5A). Ultrastructural studies (Fig. 6A) demonstrated both fibrinogen precipitates and loose platelet aggregates. Similar changes in light transmission could be produced when inert latex particles were used in place of functioning platelets. The addition of ristocetin to latex particles suspended in fibrinogen solution gave rise to aggregation curves (Fig. 5B, curve 1) almost identical to those seen with platelet suspensions; plasma was not required for this effect. Ultrastructure examination showed that the latex particles were trapped in clumps of precipitated fibrinogen (Fig. 6B). The addition of albumin to both the platelet and latex suspensions diminished both the degree of decline and subsequent increase in light transmission (curve 2, Figs. 5A and 5B).

#### DISCUSSION

In this study we have confirmed that ristocetin causes precipitation of fibrinogen as originally reported by Howard and Firkin.<sup>3</sup> In addition, we have found that albumin inhibits and low temperature facilitates this phenomenon. Under the light microscope, fibrinogen precipitates appear as fibrils resembling fibrin strands. When viewed with the electron microscope, the fibrils show no definite structure. The mechanism by which fibrinogen is precipitated by ristocetin is not known. However, fibrinogen that is resolubilized after precipitation by ristocetin can still be clotted by thrombin. It is not clear why the thrombin time is slightly shortened in samples treated with low concentrations of ristocetin. Prolongation of the thrombin time by high concentrations of ristocetin has also been observed by Meyer et al.<sup>9</sup> and is probably due to incomplete resolubilization of precipitated fibrinogen.

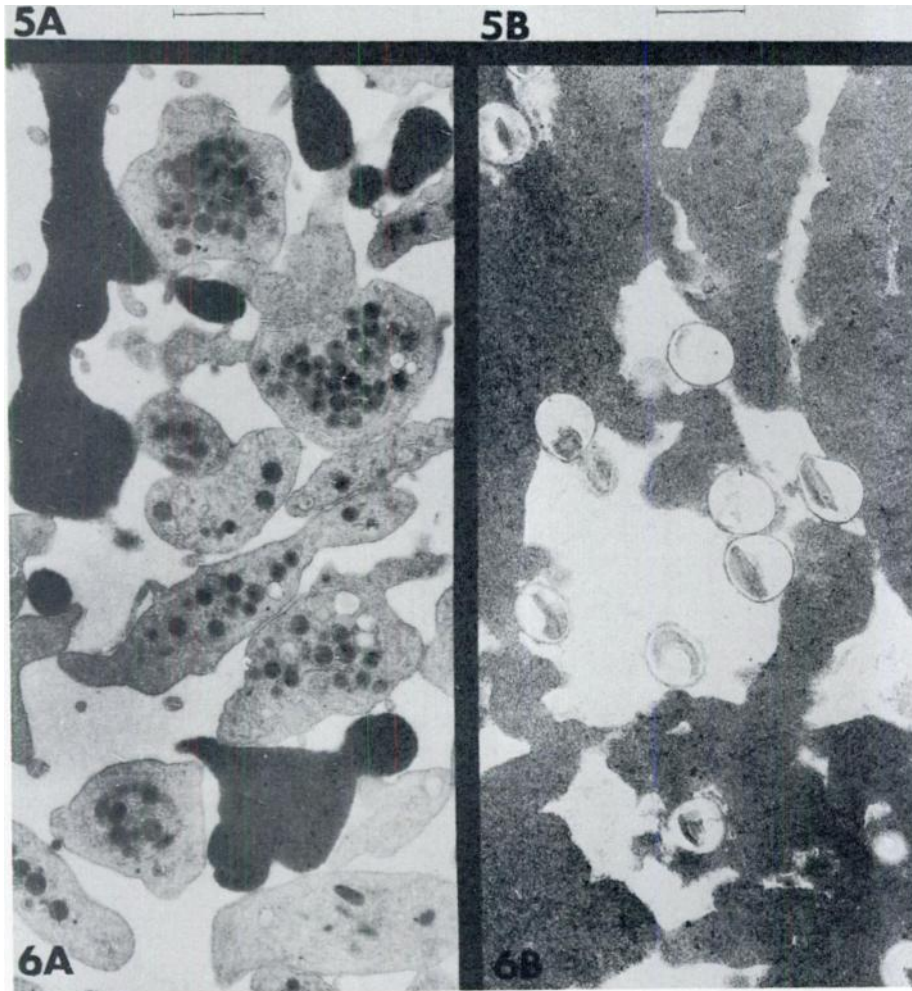
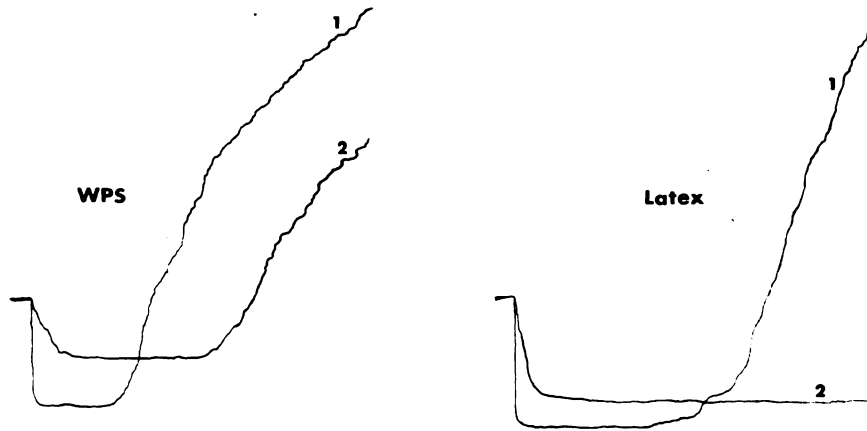
The appearance of ristocetin-precipitated fibrinogen clumps in the platelet

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**Fig. 5.** Light transmission recordings of washed platelet and latex particle suspensions exposed to ristocetin. (A) Washed platelets suspended (WPS) in a medium containing fibrinogen (300 mg/100 ml) and a small volume of plasma (curve 1). Portions of the medium also contain albumin (500 mg/100 ml, curve 2). (B) Latex particles suspended in similar medium. The suspensions were exposed to 1.75 mg/ml of ristocetin. In the absence of albumin (curve 1), similar increases in light-transmission changes have been recorded for both WPS and latex. Albumin retarded and prevented light-transmission changes in WPS and latex (curve 2), respectively.

**Fig. 6.** Electron micrographs of specimens obtained from the experiments described in Fig. 5. (A) Fibrinogen precipitates accompanied by loosely aggregated platelets.  $\times 9800$ . (B) Precipitation of fibrinogen which entrapped inert latex particles.  $\times 9600$ .





See legend on facing page.

surface cannalicular system (SCS) is consistent with the observation that there is a direct communication between platelet vacuoles and the outside environment.<sup>12,13</sup> Fibrinogen clumps in the SCS probably represent the precipitation of fibrinogen already present in the SCS.

The addition of ristocetin to latex particles suspended in fibrinogen solution generated aggregometer tracings virtually identical to those observed with platelet suspensions. Ultrastructural studies demonstrated the entrapment of the inert latex particles in clumps of precipitated fibrinogen. A plasma cofactor was not required for this effect. When ristocetin was added to washed platelet suspensions containing both fibrinogen and plasma, fibrinogen precipitates, as well as loose platelet aggregates, were formed. Thus, the changes in light transmission in this latter situation may be attributable to both platelet aggregation and precipitation of fibrinogen.

It has recently been recognized that a specific plasma factor (ristocetin aggregation factor, RAF) is deficient in the plasma of patients with severe von Willebrand's disease.<sup>3</sup> This factor has been assayed by adding the patient's plasma to a suspension of washed normal platelets and measuring the degree of aggregation produced by ristocetin.<sup>14</sup> The studies reported herein caution that, in the presence of fibrinogen, the aggregation curves produced may not only measure the activity of this plasma aggregation factor but may also nonspecifically involve platelets as entrapped particles within precipitated fibrinogen. This observation is particularly pertinent since it has recently been shown that the activity of ristocetin preparations of identical concentration may be quite variable (G. Barlow, personal communication). Therefore, diagnostic studies employing ristocetin-induced platelet aggregation should be accompanied by morphologic examinations to ensure that fibrinogen precipitation is not masquerading as platelet aggregation.

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