properdin activity not found in Fraction I appears in Fraction III. Careful definition of the variables responsible for the distribution of properdin during fractionation of plasma is being pursued.

Summary. Methods have been presented for the preparation of properdin from resin-collected and from ACD plasma by the application of the zymosan adsorption technic of Pillemer *et al.* Preliminary data are presented suggesting the feasibility of the isolation of properdin from Cohn Fraction I without the use of zymosan.

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Some Factors that Influence Utilization of Antihemophilic Activity During Clotting.* (23454)

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When normal blood or plasma clots, it loses its antihemophilic activity(1). If plasma is first adsorbed with $BaSO_4$, antihemophilic factor (AHF) remains in the adsorbed plasma even after recalcification; it thus is stabilized by the adsorption procedure. This paper describes the effect on AHF utilization of adding various plasma and serum preparations and derivatives to this stabilized plasma. It was found that only when a source of thrombin was added would AHF disappear. Within the lower range of effective thrombin concentrations, the rate of loss of AHF was proportional to the amount of thrombin available. Preliminary reports of some of these data have been made previously(2).

Materials and methods. Oxalated plasma and *native, platelet-poor plasma* were prepared as previously described(1). *PTC-deficient plasma* was obtained from a patient with a moderately severe disease characterized by hemarthrosis and hematuria. *Plasma deficient in Stuart factor* was from the patient described by Hougie and associates(3). *"Dicumarol plasma"* was prepared from a dog to whom 1400 mg dicumarol had been administered over a 13-day period. *"Stabilized plasma"* was made by twice adsorbing oxalated plasma with $BaSO_4$ (Merck R; 100 mg/ml at 28°C for 20 min). *Normal and hemophilic canine sera* were obtained from blood allowed to stand in glass for 24 hours. *Serum accelerator factor (SAF)* was prepared as described earlier(4). *Plasma thromboplas-*

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TABLE I. Effect of Various Plasmas, Sera, or Fractions on AHF in Stabilized (Adsorbed) Plasma.

Exp. No.	Material tested	% prothrombin in test material	Residual AHF as % initial level		
			Reaction period in min. 5	15	30
I. Controls					
1	Oxalated saline (mean of 9 tests)	0	—	—	98 ± 11.7
2	Normal canine plasma (" " 4 ")	100	11 ± 6.2	8 ± 4.8	<5
3	" human " (" " 3 ")	100	<5	8 ± 4.6	<5
II. Normal serum and serum fractions					
4	Normal canine serum	<5	95	82	70
5	Canine SAF (100 Alexander U/ml)	<5	84	89	97
6	Adsorbed canine serum	<5	—	97	93
7	Human PTC preparation	<5	88	82	82
III. Deficient plasmas and sera					
8	Dicumarol canine plasma	<5	98	97	97
9	Hemophilic " "	105	<5	<5	<5
10	PTC-deficient human plasma	115	<5	<5	<5
11	Hemophilic canine serum	18	17	<5	<5
12	Stuart-factor deficient human plasma	86	39	22	12

tin component (*PTC*) was made by the method of White, Aggeler, and Glendening (5). The *prothrombin* used was adsorbed from canine oxalated plasma with $Al(OH)_3$ and eluted with phosphate buffer; it contained 3000 Iowa units/mg N. *Thrombin* was obtained by activating a canine prothrombin preparation with 25% sodium citrate(6); it contained 5000 units/mg N. *Bovine AHF* was made by a modification of Bidwell's procedure(7) and was diluted to approximate the activity of canine plasma. *Oxalated saline* consisted of 1 part 0.1 M sodium oxalate in 99 parts saline (0.15 M NaCl). *Crude cephalin* was made from dog brain(8). The two-stage method of prothrombin determination(9) was used. Assays of AHF were performed by the method of Langdell, Wagner and Brinkhous(8).

Procedure. The following *test system* for studying AHF utilization was employed. All plasma and test materials were first dialyzed against oxalated saline for 2 hours at 4°C with constant agitation. Reagents were mixed in the following order and amounts: 1.4 ml stabilized plasma, 0.1 ml imidazole buffer (pH 7.3), 0.1 ml 0.3% cephalin, 0.1 ml test material, 0.24 ml 1.2% $CaCl_2$. After varying reaction periods up to 30 minutes, 0.4 ml 0.1 M sodium oxalate were added and residual AHF assayed after adsorption with $BaSO_4$. Antihemophilic activity at zero time represented 100% AHF. Slight variations in this basic procedure are indicated in the text. Whenever reagents were omitted, oxalated saline was substituted to maintain constant volume.

Results. The effect on AHF utilization of adding various plasmas and sera to the test system is shown in Table I. Illustrative experiments only are given. Exp 1 demonstrates that AHF is stable in adsorbed plasma, even in the presence of cephalin and calcium. In another experiment of this type, not included in the table, 109% AHF was found after a reaction period of 6 hours. The rapid disappearance of AHF caused by only a small amount of canine or human plasma is illustrated by Exp 2 and 3. When normal canine serum or its fractions were tested (Exp 4, 5, and 6), little or no loss occurred. The same relative stability was encountered when a human PTC preparation (Exp 7) or dicumarol plasma (Exp 8) was added. None of the materials tested in Exp 4-8 contained sufficient prothrombin to be detected in the 2-stage assay. Hemophilic plasma (Exp 9) and plasma which was moderately deficient in PTC (Exp 10) were as effective as normal plasma in causing loss of antihemophilic activity; they contained normal amounts of prothrombin. In contrast to normal serum (Exp

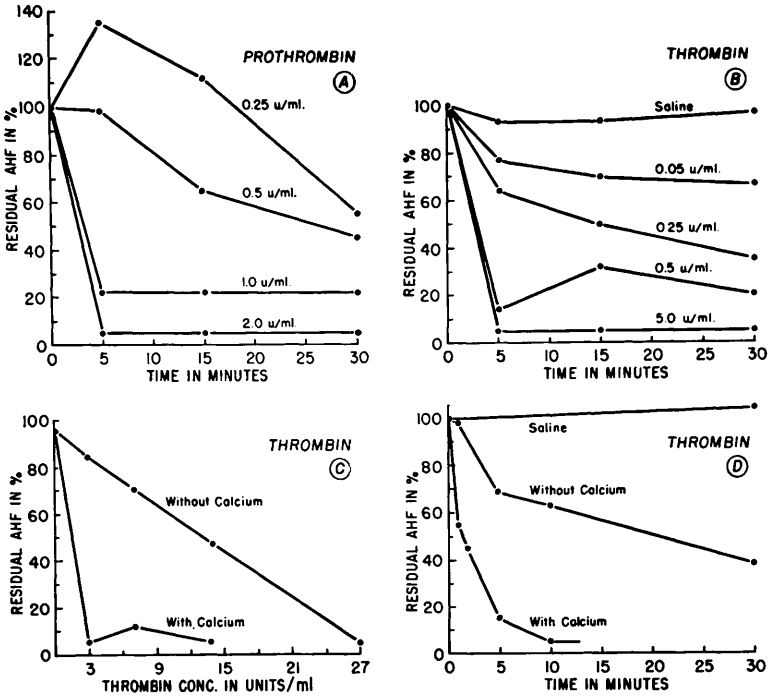

FIG. 1. Effects of purified prothrombin and thrombin on AHF in stabilized plasma. Procedures performed at 28°C in silicone-treated tubes. Indicated concentrations refer to final reaction mixtures. See text for complete test system. A. Effect of various concentrations of purified prothrombin (indicated for each curve) on AHF utilization in the presence of calcium and cephalin. B. Effect of various concentrations of thrombin (indicated for each curve) on AHF utilization with calcium but not cephalin present. C. Comparison of effects of various concentrations of thrombin on AHF utilization with and without added calcium. Reaction time = 30 min. Saline control (no thrombin) contained 95% AHF after 30 min. D. Comparison of AHF utilization rates in the presence of a constant concentration of thrombin (5 u/ml) with and without added calcium.

4), hemophilic serum (Exp 11) produced prompt AHF loss; it contained 18% residual prothrombin. Thus, only those reagents that contained prothrombin effected a loss of antihemophilic activity under these conditions.

Even when prothrombin was present, unless it was readily converted to thrombin, the AHF persisted at high levels. Human plasma deficient in the Stuart factor formed thrombin slowly; although it contained prothrombin, only 54% was converted in 1 hour(3). There was a correspondingly slow drop in the AHF (Exp 12). To show further that AHF loss is related to conversion of some prothrombin to thrombin, both AHF and prothrombin were repeatedly determined in slowly clotting, platelet-poor plasma at 4°C. Under these conditions, both factors were relatively stable. In one experiment, after 2 hours, 103% prothrombin and 120% AHF were present. A flimsy fibrin clot then formed. During the next 30 minutes only 23% of the prothrombin was utilized, but all of the AHF disappeared.

The effects of purified prothrombin and thrombin on the rate of AHF loss were studied next (Fig. 1). Fig. 1A demonstrates the results of adding various concentrations of purified prothrombin to the test system. Increasing the amount of prothrombin caused increasingly rapid loss of AHF. As little as 2 units/ml caused all of the AHF to be lost in 5 minutes. Cephalin and calcium were necessary constituents of these reaction mixtures; without them, the prothrombin was not converted and no loss of AHF occurred. In the experiments shown in Fig. 1B and 1C, the loss of AHF from stabilized plasma was proportional to the thrombin concentration. Cephalin was found to be unnecessary when this preformed thrombin was used, and was

omitted from these mixtures. Without ionic calcium, more thrombin was needed to cause a given loss of AHF (Fig. 1C). Even so, there was a linear relationship between the concentration of thrombin and the AHF lost. When calcium was added to the thrombin-plasma mixtures, all of the AHF disappeared with a thrombin concentration as low as 3 units/ml. Relative rates of AHF loss with a constant amount of thrombin in the presence and absence of ionic calcium are compared in Fig. 1D. Progressive loss of antihemophilic activity occurred in the absence of calcium, but the reaction was markedly accelerated when calcium was available.

Fibrinogen was present in the reaction mixtures of each of the above experiments. Other experiments were done in which fibrinogen was reduced to trace amounts. In one, purified bovine AHF was substituted for stabilized plasma and thrombin was added as in Fig. 1D. The rates of disappearance of antihemophilic activity were similar to those obtained with stabilized plasma. Without added calcium, 56% AHF was lost in 30 minutes; with calcium present, all activity disappeared in less than 10 minutes.

Discussion. These data suggest that thrombin plays a major role in utilization of AHF during clotting. That some factor which is adsorbable onto $BaSO_4$ is required is demonstrated by the stability of AHF in adsorbed plasma after recalcification. This factor appears to be diminished in dicumarol plasma, normal serum or preparations of SAF or PTC, all of which are poor thrombin sources. Plasmas or sera that contain prothrombin, however, whether normal, hemophilic or deficient in PTC or Stuart factor, will restore conditions that permit the AHF to disappear when cephalin and calcium ions are available to cause its conversion to thrombin. Preformed thrombin is able to cause the loss of antihemophilic activity from stabilized plasma or from purified AHF. Perhaps AHF is another molecule subject to the enzymatic splitting action of thrombin. Only small amounts of thrombin are required to inactivate AHF if calcium ions are available, a finding in keeping with our earlier impression that prompt and thorough decalcification is necessary for optimal preservation of AHF in stored blood (10). Commercial preparations of thrombin have been reported to be contaminated with profibrinolysin(11). Fibrinolysin can destroy AHF(12). It would seem unlikely that the thrombin preparation used in these experiments (prepared by adsorption, elution and citrate-activation) had significant fibrinolytic activity. However, effects of possible contaminants of the purified reagents cannot be completely excluded.

Reduction in the levels of circulating AHF has been observed in dogs subjected to cold-injury(13) and thromboplastin injection(15), two conditions that appear to be associated with systemic coagulation. The *in vivo* response to AHF appears to be a sensitive index of intravascular clotting, probably because only a small amount of thrombin is required to cause loss of antihemophilic activity. These conclusions are further supported by the depressions in circulating AHF that have been produced by injecting thrombin preparations intravenously(14,15).

There is some discrepancy between our observations and those made by Douglas(16). He observed a normal rate of disappearance of AHF in the absence of platelets; our experiments indicate a retarded rate if the platelets are sufficiently reduced to delay thrombin formation. This contrast might be explained by differences in methods. A similar explanation probably applies to variations between our data and those of Johnson and Seegers(17). These authors did observe loss of activity of "platelet cofactor I" in the presence of thrombin and calcium, but none if calcium were omitted from the reactions.

It is perhaps possible that AHF passes through a more active phase under the influence of thrombin. Such a concept would be in keeping with an autocatalytic role of thrombin and the formation of a "prothrombin conversion factor" as recently described(18). In Fig. 1A, it will be noticed that the most dilute thrombin elicited a brief hyperactive phase. In other experiments, this hyperactivity was of even greater magnitude, but was evanescent and difficult to reproduce consistently.

Summary. A study has been made of the effects of various plasmas, sera and fractions

on AHF in stabilized ($BaSO_4$-adsorbed) plasma. Only reagents that provided a source of thrombin caused AHF to disappear after recalcification. Materials tested that produced loss of AHF were: normal, hemophilic, PTC-deficient and Stuart factor deficient plasmas and hemophilic serum. All contained prothrombin. Those that produced no significant loss were: dicumarol plasma, normal serum, SAF, PTC and adsorbed serum; they contained no detectable prothrombin. Slow prothrombin conversion (*e.g.* Stuart plasma, platelet-poor plasma) was accompanied by slow AHF utilization. Removal of fibrinogen from reaction mixtures did not abolish AHF loss. Purified prothrombin caused utilization of AHF in proportion to its concentration if cephalin and calcium ions were present to cause conversion to thrombin. Preformed thrombin was also effective in proportion to its concentration; its effect was accelerated by calcium ions. The significance of these findings is discussed in relation to blood preservation, intravascular coagulation and basic clotting reactions.

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Serial Propagation of the Guinea Pig Salivary Gland Virus in Tissue Culture. (23455)

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The use of tissue culture methods for isolation and propagation of the viruses of mouse(1) and human(2-4) salivary gland disease and the attendant development of technics for their serological study suggested similar efforts to establish the salivary gland virus of guinea pigs. Andrewes(5) found that the inoculation of suspensions of infected salivary glands into modified Maitland cultures of guinea pig testis regularly resulted in development in 3 to 6 days of intranuclear inclusion bodies in mononuclear cells in the in-