

Qualitative Description of Factors Involved in the Retraction and Lysis of Dilute Whole Blood Clots and in the Aggregation and Retraction of Platelets

FLETCHER B. TAYLOR, JR., and HANS J. MÜLLER-EBERHARD

From the University of Pennsylvania, Philadelphia, Pennsylvania 19104 and Scripps Institute, LaJolla, California 92037

ABSTRACT Dilute whole blood clots were prepared by addition of thrombin to blood diluted 1:10 in phosphate buffer. The pH of this buffer was 7.4 and the ionic strength was 0.084. Though the ionic strength was low, there was no hemolysis of red corpuscles due to the contribution to the osmotic gradient by plasma salts and proteins. In the standard assay the clot was formed by addition of thrombin at 4°C then incubated at 37°C. Retraction and lysis of these clots were inhibited by removal of platelets and by increasing concentrations of purified thrombin. Retraction and lysis were also inhibited by inactivation of any one of the following factors: γ M globulin, complement components C4 and 3, and (in the case of lysis) plasminogen.

Evidence that some of the above serum factors were adsorbed to the platelet membrane was obtained by aggregation of washed platelets by antisera to these factors (i.e. fibrinogen, γ M, and C4 or C3). These platelets were not aggregated by antisera to other serum proteins (by albumin, transferrin, γ G globulin).

These and other studies suggested that platelets, thrombin, fibrinogen, γ M globulin (cold agglutinin), complement components, and plasminogen influenced and facilitated retraction and lysis of clots. These studies also suggested that platelets and some of these factors were physically associated.

Because of this physical association, and because of the fact that clot retraction is associated with aggregation and retraction of platelets, we extended the above observations to include a study of the effect of these same serum factors on serum-induced aggregation and retraction of washed platelets. (Other terms which have been in use in the past to describe serum-induced platelet aggregation and retraction have included those such as

platelet "fusion" and "viscous metamorphosis," neither of which fully described the phenomena.)

Platelet aggregation and retraction induced by serum was markedly accelerated by addition of increasing concentrations of thrombin and (or) cold agglutinin. Hirudin and antisera to γ M globulin inhibited serum-induced aggregation and retraction of platelets. Reconstitution of inactivated serum with purified C4, 3, and 5 and thrombin restored its capacity to induce aggregation and retraction of platelets.

Therefore, we postulated that platelet aggregation and retraction were necessary for clot retraction and that platelet aggregation and clot retraction facilitated clot lysis. More specifically we postulated that thrombin, in addition to catalyzing clot formation, also modified the platelet membrane such that γ M globulin (cold agglutinin) and complement components can act on the platelet membrane leading to (a) aggregation and retraction of the platelets, (b) retraction of the clot, and (c) to the activation of plasminogen either on the surface of the platelet by C8i and (or) by release of platelet activators of plasminogen.

INTRODUCTION

Little is known about the mechanisms by which blood clots or fibrin monolayers normally lyse following activation of the coagulation system. Because it has been assumed that the enzyme plasmin (fibrinolysin) plays a key role in this mechanism, plasmin, its substrates fibrinogen and fibrin, and its natural inhibitors all have been subjected to extensive study and characterization (1). The biologic importance of these studies has rested on the assumption that the plasmin system serves to maintain a homeostatic balance between the polymerization of fibrin and the dissolution of this polymer. Although much is known about the activation of the sys-

Received for publication 29 December 1969 and in revised form 22 June 1970.

tem which polymerizes fibrinogen, little information is available regarding the subsequent activation of the system which hydrolyzes fibrin in the intact blood clot. We propose to describe in qualitative terms some of the factors and events which culminate in lysis of the intact blood clot (i.e. platelet aggregation and retraction, clot retraction, and clot lysis). Thus, in the broadest sense, the purpose of this paper is to describe some of those events which occur within the clot after the activation of thrombin and which lead to the activation of plasminogen.

METHODS

Partially purified 19S cold agglutinin was prepared from the serum of a patient (E.R.) with a 1:5000 titer of polyclonal 19S cold agglutinin according to the method described by Nehrota and Charlwood (2). γ M macroglobulin and cryoglobulin were obtained from patients with Waldenström's macroglobulinemia and multiple myeloma respectively. Highly purified complement components C1q, C3, C4, and C5 respectively were prepared according to methods described in references 3-6. Partially purified C8 and C8i were prepared according to methods to be described elsewhere. These preparations were stored in 0.2 ml aliquots (200 μ g/0.2 ml) at -60°C .

Cobra factor was prepared from crude cobra venom (Sigma Chemical Co., St. Louis, Mo.) by extensive dialysis of 400-mg amounts (solubilized in 10 ml of 0.01 M Tris buffer) against 2000 ml of 0.01 M Tris buffer, pH 8.5 at 4°C overnight. The dialysate was then adsorbed on triethylaminoethane cellulose (TEAE) with a binding capacity of 0.93 mEq/g which had been equilibrated with 0.01 M Tris buffer, pH 8.5 at 4°C and then packed in a 1×80 cm column. The adsorbed material was eluted with a linear salt gradient (0.05-0.25 μ). The mid-portion of the third of three peaks was then concentrated by ultrafiltration, dialyzed, rechromatographed, concentrated again, and then passed through Sephadex G-100 (25 \times 100 cm column) which had been equilibrated with 0.01 M Tris-0.51 M NaCl buffer, pH 8.5, 4°C . The second of three peaks was pooled, concentrated by ultrafiltration, and stored at -60°C . The activity of 0.05 ml aliquots of 1:1, 1:2, 1:4, etc. dilutions of the various fractions from the chromatographic steps and of the purified cobra factor was determined by monitoring conversion of the β 1c globulin to β 1a globulin in 0.25 ml of fresh serum over a 30 min period at 37°C . This conversion was monitored by immunoelectrophoresis of the serum in 1% agar gel which was made up in the standard buffer plus 0.01% ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) (7). This purified cobra factor migrated as a single band on acrylamide gel. It inactivated only the third component of complement. It did not affect the activity of any of the other complement components, thrombin, γ M globulin, or plasminogen. Any effect that cobra factor might have had on these proteins was monitored by observing changes in electrophoretic mobility on acrylamide gel (8), and in the case of thrombin and plasminogen by changes in specific activity employing assays using fibrinogen (9) and casein (10) substrates respectively. This material was stored in 0.2 ml aliquots (300 μ g/0.2 ml) at -60°C .

Highly purified plasminogen (11) and streptokinase (SK) (12) were prepared according to methods described elsewhere. The specific activity of the plasminogen and strep-

tokinase were 25 casein U/mg of protein and 11.2 SK LMe U/mg N respectively (12). These were stored in 1 ml aliquots (0.4-1 mg/ml) at -60°C .

Bovine thrombin (200 mg) obtained from Parke, Davis & Co., Detroit, Mich., was dialyzed overnight against 0.002 M glycine buffer, pH 9.0, 4°C , and then adsorbed on TEAE which, after equilibration in 0.002 M glycine buffer, pH 9.0, 4°C , had been packed in a 1.5×80 cm column. The adsorbed material was then eluted with a linear salt gradient (0.01-0.6 μ). The mid-portion of the first of three peaks was pooled, concentrated by ultrafiltration, and then filtered through Sephadex G-100 which had been packed in a 2.5×100 cm column and equilibrated with 0.001 M phosphate buffer plus 0.3 M NaCl, pH 7.4, 4°C . The descending portion of the first of three peaks was then assayed (9) with other fractions and stored in 1 ml aliquots (0.4-0.6 mg/1 ml) at -60°C . The specific activity of this partially purified thrombin was 300-450 NIH U/mg N. Plasminogen-free human fibrinogen was prepared from plasma according to the method of Brown and Rothstein (13). The fibrinogen was 98-99% clottable and was stored in 1 ml aliquots (10 mg/ml) at -60°C . All the above proteins were assayed for plasminogen on bovine fibrin clots.

Rabbit antisera to these proteins were prepared as described previously (3, 5, 6). Rabbit antisera to human albumin, α_2 -macroglobulin, fibrinogen, transferrin, and γ G and γ M globulin were obtained commercially from Behringwerke Company, Marburg/Lohn, Germany. The partially purified globulin fractions of these antisera were prepared as described by Kabat and Mayer (14).

RESULTS

Relationship of platelets to clot retraction and lysis.
The basic assay for monitoring clot lysis was first described by Fearnley, Bolmforth, and Fearnley (15). 1 ml of whole blood was drawn from the anticubital vein of the forearm into a plastic syringe. This was immediately diluted 1:10 in phosphate buffer (7.576 g $\text{Na}_2\text{HPO}_4 + 1.616$ g $\text{KH}_2\text{PO}_4/1000$ ml, ionic strength 0.084, pH 7.4) at 4°C . At this dilution and temperature the blood will not clot spontaneously. It was at this point that the blood was manipulated (i.e. centrifuged, sonicated) or specific modifying agents added (i.e. antisera, cobra factor, etc.). The diluted whole blood was then transferred in 2-ml aliquots to 15×100 mm test tubes and coagulated at 4°C with 1.0 U of thrombin. After 20 min at 4°C , the formed clots were transferred to a 37°C water bath and observed for retraction and shedding of red cells $1\frac{1}{2}$ hr after transfer. They were observed for lysis every $\frac{1}{4}$ hr applying the criteria described by Fearnley et al. (15). The lysis times of blood drawn between 8:00 and 10:00 a.m. from the four normal fasting subjects (all males between the ages of 30 and 40) used in these studies was $4 \pm \frac{1}{2}$ hr. All the studies reported herein were done on blood from these same four subjects. All studies included unmodified (control) samples in triplicate and modified (experimental) samples in duplicate. The lysis times of the triplicate unmodified (control) samples varied from each other by no

TABLE I
Relationship of Red Blood Cell and Platelet Counts to Clot Dissolving Time*

Centrifugation of diluted blood for 10 min at 4°C	Clot dissolving time		Clot retraction 0-4+	Supernatant cell counts		
	Supernatant alone	Remixed samples		Platelet count/mm ³ (×10 ⁹)‡	Red blood cells/mm ³ (×10 ⁹)	White blood cells/mm ³ (×10 ³)
<i>g</i>	<i>hr</i>	<i>hr</i>				
0	4.0	4.0	4+	25.0	513.3	5.20
200	4.0	4.0	4+	18.2	0.26	0.80
600	4.0	4.0	4+	11.1	0.24	0.09
1200	4.0	4.0	4+	8.1	0.29	0.02
1800	24	4.0	1+	1.1	0.05	0.01

Clot retraction is graded 0 to 4+. 0 indicates no retraction and 4+ indicates that the clot has retracted to $\frac{1}{4}$ or less of its original volume as determined by inspection.

* Average values from studies on five healthy adults at 9:00 a.m.

‡ Platelet counts carried out with a phase microscope.

more than 15 min. All glassware used in these studies was siliconized.

The question regarding requirement of platelets for lysis of these clots was studied by removing them by differential centrifugation (16) or sonication. In the centrifugation studies, 4-ml aliquots of the diluted whole blood were centrifuged at 0, 200, 600, 1200, and 1800 *g* at 4°C for 10 min. The resulting supernatants were then transferred and white cell, red corpuscle, and platelet counts were made. 2-ml aliquots of these supernatants were then coagulated at 4°C and then handled and observed as described above.

In the studies in which platelets were sonicated, 4-ml aliquots of platelet-rich plasma obtained by centrifuging the diluted whole blood at 500 *g* for 10 min were sonicated at 60 kilocycles/sec with a Lourdes sonicator for 10 min at 4°C. 2-ml aliquots of this material were then coagulated and observed as described above. In addition, 2-ml samples of platelet-rich plasma which already had been coagulated over a 10 min period at 4°C were similarly sonicated. The number of platelets per mm³ before and after sonication were counted with a phase microscope. Destruction of platelets within the formed clots was estimated by comparing photomicrographs of sonicated vs. control diluted whole blood or plasma clots which were prepared as described by James, Johnson, Monto, Diab, and Caldwell (17).

Table I shows the effect of removal of platelets by centrifugation on clot retraction and clot lysis. The results shown are an extension of a preliminary study reported previously (16). There was little appreciable effect on clot retraction or change in lysis time upon removal of most of the red and white cells at 200 *g*. However, when the platelet count was reduced to 1000/mm³, retraction was reduced and clot lysis was prolonged. Resuspension or addition of washed platelets in a

duplicate set of centrifuged samples yielded clot lysis times identical with the uncentrifuged controls. Sonication of diluted platelet-rich plasma before the addition of thrombin resulted in no clot retraction and lysis times of over 14 hr vs. 4+ clot retraction and lysis times of 4 hr in control samples (provided count of intact platelets was reduced below 1000/mm³). Sonication of the formed dilute platelet-rich plasma clot resulted in platelet destruction with minimal distortion of the normal clot structure as determined by examination of photomicrographs. Clot retraction and lysis did not differ from the control samples provided the sonication was done after formation of the clot was complete.

Relationship of thrombin and serum factors to clot retraction and lysis. The effect of thrombin on clot retraction and lysis was examined by adding 0.1, 0.2, 0.4, 0.8, 5, and 10 NIH U of thrombin both to the usual clot system coagulated at 4°C then changed to 37°C, and to a clot system kept at 37°C throughout. Varying concentrations of thrombin under the former conditions had little effect on clot retraction or lysis, whereas under the latter conditions (37°C throughout, Table II) lower

TABLE II
Effect of Varying Concentrations of Thrombin on Retraction and Lysis of Clots Formed at 37°C

Thrombin concn	Retraction (0-4+)	Lysis
<i>NIH U</i>		<i>hr</i>
0.1	4+	7½
0.2	4+	18
0.4	4+	24
0.8	3+	36
5.0	2+	48
10.0	2+	48

concentrations of thrombin enhanced both clot retraction and lysis. None of the supernatants from clots formed under these conditions had any residual clottable fibrinogen as determined by addition of 10 U of thrombin.

The question regarding the role of γ M, complement components C3 and C4, and plasminogen in retraction and lysis of dilute whole blood clots was studied indirectly by addition to the standard clot lysis assay of partially purified γ -globulin fraction of rabbit antisera directed against these factors (18). The γ -globulin fractions used were reconstituted to the original serum volume and dialyzed against 1000 ml of phosphate buffer for 18 hr to remove $(\text{NH}_4)_2\text{SO}_4$ salts. 0.25 ml aliquots of these preparations were then added to the 2-ml aliquots of diluted whole blood at 37°C. After 5–10 min the samples were placed in an ice bath, thrombin was added, and the samples were handled and observed as described above. Control samples included the γ -globulin fraction of normal rabbit serum and that of rabbits immunized against human albumin, α_2 -macroglobulin, transferrin, and γ G globulin.

The question regarding the specificity of inhibition caused by certain of the above antibodies was studied by addition of 20–40 μ g of homologous antigen (i.e. purified γ M, C3, C4, and plasminogen) to the dilute whole blood samples before addition of the antibody and thrombin. As a control these purified proteins were also added in the same quantities to the clot lysis assay to which no antibodies were added.

The effect of specific antibodies, and their respective antigens, on clot retraction and lysis is included in Table III. The results shown are an extension of a preliminary study reported previously (18). This table shows that antibodies directed against γ M globulin, C3, C4, and plasminogen were the most effective inhibitors of clot retraction and lysis, whereas antibodies against albumin, α_2 -macroglobulin, transferrin, and γ G globulin did not affect clot retraction or lysis. Addition of 20–40 μ g of homologous antigen (i.e. purified γ M, C4, C3, or plasminogen) to the above antibody-containing systems maintained normal clot retraction and lysis provided that they were added before the antibody and thrombin. Addition of C3 and C4 alone to the standard assay failed to reduce the time of lysis below 3 hr. The effect of inactivation of C3 by cobra factor on the retraction and lysis of dilute whole blood clots was studied by addition of 10–100 μ g of purified cobra factor to the clot lysis system at 37°C. After 10 min the temperature was lowered from 37° to 4°C and thrombin (1 U) was added. The system was then handled and observed as described above. This experiment was then repeated but *after* the 10 min incubation step (37°C) with cobra factor, the blood was cooled to 4°C and 100–300 μ g of fresh C3 was added. Thrombin was then added, and

TABLE III
Effect of Specific Antibodies to Human Serum Proteins; Cobra Factor, γ M Globulins (19S Macro, Cryo, and Cold Agglutinins), and Temperature on Retraction and lysis of Blood Clots

Inhibiting substance	Retraction (0–4+)	Lysis time hr
Phosphate buffer	4+	4
Anti- γ M globulin	0	24
Anti-C3	2+	14
Anti-C4	2+	12
Anti-C3 and -C4	1+	24
Anti-plasminogen	3+	24
Anti-albumin	4+	3
Anti- α_2 -macroglobulin	4+	4
Anti-transferrin	4+	4
Anti- γ G globulin	4+	3
Cobra factor (10 μ g)	2+	12
Cobra factor (10 μ g) and then C3	4+	4
C8	4+	4
C8i	4+	1
Assay run at 4°C until clot forms then 37°C	4+	4
Assay run at 37°C throughout	2+	24
19S cold agglutinin in 37°C system	3+	8
19S cryoglobulin in 37°C system	1+	24
19S macroglobulin in 37°C system	1+	24

after the clot was formed at 4°C the system was observed for lysis in the usual manner. Table III also shows that cobra factor which inactivates C3 also inhibited clot retraction and lysis. This effect of cobra factor was reversed by addition of an excess of C3 substrate at the end of the inactivation period (just before the addition of thrombin).

In order to determine more specifically and directly what effect C8 and hemolytically inactive C8i had on clot retraction and lysis, the following studies were done. 0.1 ml aliquots (50 μ g) of partially purified C8 and C8i from the same batch were added to 2-ml aliquots of diluted whole blood at 4°C followed 10 min later by thrombin (1 U). This system was then observed as described above. C8i is a hemolytically inactive form of C8 which on occasion has been found to appear spontaneously during the last step of the chromatographic isolation of C8. Though this material is hemolytically inactive, it is immunologically identical with C8 and, as described below, it has a profound effect on the behavior of the clot lysis and platelet systems. Table III shows that addition of C8i to this system resulted in very rapid times of lysis (1 \pm $\frac{1}{2}$ hr), whereas addition of C8 did not affect lysis.

In order to determine more specifically which component of the γ M family of proteins might be involved

in this phenomena, we studied the effect of 19S macroglobulin, 19S cryoglobulin, and polyclonal 19S cold agglutinin on (a) the standard clot lysis assay in which the diluted whole blood was clotted at 4°C in the usual manner and (b) the standard clot lysis assay in which the diluted whole blood was clotted at 37°C. 0.25 ml aliquots of the above proteins (50–300 µg/0.25 ml) were added to 2-ml aliquots of the diluted whole blood kept at either 37°C or 4°C. 10 min later 1 U of thrombin was added and the samples were handled and observed as described above. Table III shows that addition of purified 19S cold agglutinin to the standard clot lysis system which was then coagulated at 4°C had no effect on the normal clot retraction or lysis. However, addition of cold agglutinin to the clot lysis system which was then coagulated at 37°C increased clot retraction from 1–2+ to 4+ and shortened the lysis time from 18 ± 2 hr to 8 ± 1 hr. Thus, the cold agglutinin added to the system kept at 37°C appeared to obviate the need for coagulating the system at 4°C. The clots which were formed at 37°C instead of 4°C retracted and lysed normally, provided sufficient cold agglutinin was added to the system. In contrast to the effect of 19S cold agglutinin, it was observed that neither 19S macroglobulin, 19S cryoglobulin, nor monoclonal 19S cold agglutinin obtained from Dr. A. G. Cooper produced this effect.

Because of the possibility that the γ M proteins (19 s cryoglobulin, macroglobulins, and cold agglutinins) or the complement components (C3, C4, C8, and C8i) might be contaminated with trace amounts of plasminogen, plasmin, or thrombin, they were studied for streptokinase-activatable fibrinolytic and thrombin activity in the following manner.

0.1 ml aliquots (100 µg) of each of these proteins was added to 0.5 ml of 0.2% fibrinogen solubilized at 22°C in 1×10^{-3} M CaCl—0.01 M Tris buffer, pH 7.4) followed by 0.1 ml of streptokinase (0.3 µg, 70 U) and 0.1 ml of bovine thrombin (1 U). The fibrin clots which formed were then incubated at 37°C and observed every

5 min for lysis. The samples were run in duplicate. Controls consisted of fibrinogen clotted with 1 U (0.1 ml) of thrombin, fibrinogen clotted with 1 U of thrombin to which 0.1 ml (0.3 µg) of SK had been added, and fibrinogen with 0.1 ml of each of the above proteins to be tested. This last control was to test these proteins for thrombin activity. In the case of C8i, assays included C8 and C8i alone, with plasminogen, or streptokinase.

None of these reagents had fibrinolytic activity either alone or upon addition of streptokinase in a purified thrombin-plasminogen-fibrinogen assay system. None of these reagents had thrombin activity. However, C8i but not C8 partially activated plasminogen. C8i alone plus fibrinogen and thrombin significantly decreased the rate of fibrin polymerization but did not hydrolyze fibrin.

Relationship between the pH and temperature of the reaction system and clot retraction and lysis. The pH at which there was an optimum rate of clot retraction and lysis was determined by adjusting the pH (with 0.1 M HCl) of separate 20-ml aliquots of diluted whole blood to pH 5.5, 6.0, 6.5, 7.0, and 8.0 and then determining the times of clot lysis of 2-ml aliquots as described previously. In addition, the relation between pH and temperature was studied. Two aliquots of diluted blood, A and B, were adjusted to pH 7.4 and 6.5 respectively. Two triplicate sets of samples from the aliquot A pool were then coagulated at 37°C and at 4°C respectively and then observed for clot retraction and lysis at 37°C. The same was done with aliquot B.

Fig. 1 shows the time of clot lysis as a function of pH. The optimum pH lies between 6.0 and 6.5 which is identical with the optimum pH range for complement activity. This is also the optimum pH for adsorption of cold agglutinin to red cell membranes (19). In this regard it is also of interest to note that at pH 6.5 these clots retracted and lysed normally when the temperature was kept at 37°C throughout, thus obviating the necessity of forming the clots at 4°C before incubating them at 37°C. Thus, conditions favoring clot retraction and lysis can be produced either by low temperatures at neutral pH or higher temperatures at pH 6.5.

Physical relationship of the serum factors to platelets. In order to examine the possibility of an association between serum factors and platelets, platelets were harvested, washed, and examined for agglutination upon addition of specific antisera directed against the proteins in question (18). The platelets were harvested from 10 ml of whole blood diluted 1:10 in phosphate buffer at 4°C (identical with that used in the clot lysis assay) by centrifuging them at 500 g for 10 min in a Sorvall PR2 centrifuge and then collecting the platelet-rich supernatant. These platelets were then washed thrice in the same phosphate buffer and resuspended in buffer to

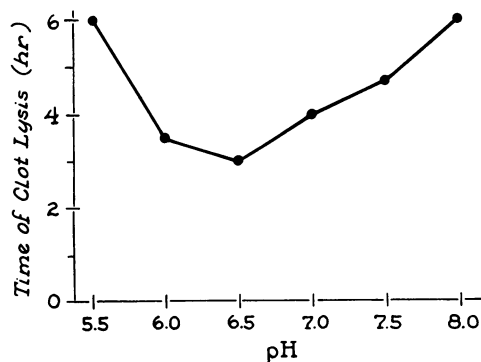


FIGURE 1 Plot of time (hours) of clot lysis vs. pH.

give a suspension of 3×10^8 platelets per mm^3 . Plastic centrifuge tubes were used in all the above steps. The yield of intact unaggregated platelets was approximately 10%. Since we wished to examine the platelets under conditions identical with those of the assay of clot lysis, no chelating agent was used. In a parallel study the platelets were also harvested employing a chelating agent as described later. The effect of various reagents (i.e. 200 $\mu\text{g}/\text{ml}$ of 19S cold agglutinin, 19S cryoglobulin, and macroglobulin; antisera to fibrinogen, γM , C3, C4, plasminogen, albumin, α_2 -macroglobulin, transferrin, and γG globulin) on these platelets was examined by adding 0.05 ml of various dilutions of each of these reagents to 0.1 ml of the above platelet suspension ($3 \times 10^8/\text{mm}^3$) in the separate wells of an agglutination plate. These preparations were placed in a shaker at room temperature for 30 min and then examined for agglutination with a phase microscope. All determinations including controls were run in duplicate.

The specificity of the agglutination phenomena was studied by addition of the purified homologous antigens (50 $\mu\text{g}/0.5$ ml of antisera of fibrinogen, γM , C3, C4) to their respective antisera before addition to platelets. The degree of agglutination was recorded and compared with that observed when antiserum alone was added to the platelets.

We found that 1:10 dilutions of anti-fibrinogen, anti- γM , anti-C3, and anti-C4 sera caused a 2-4+ agglutination of platelets, whereas antisera to C8, plasminogen, albumin, α_2 -macroglobulin, and transferrin caused no agglutination. The antisera to γG or γM globulins caused 0 and 2-3+ agglutination respectively. Purified 19S macroglobulin, C3, and C4 inhibited agglutination when added to the respective antisera before mixing with platelets. We also found that purified 19S cold agglutinin caused 3+-4+ agglutination of platelets whereas 19S cryoglobulin or macroglobulin did not cause agglutination.

Functional relationship of C3 to platelets. Immune adherence to erythrocytes is known to depend on the presence of C3 specifically bound onto the surface of the adhering particle (20). Assuming that the same conditions would apply to platelets, the presence of specifically bound C3 on platelet membranes was studied by incubating 2 ml of 1×10^8 washed platelets with 0.1 ml of $1 \times 10^8/\text{ml}$ red blood corpuscles from the same subject. This was done according to the method described by Nishioka (21). Consistent 2-3+ reactions were obtained between washed platelets with bound C3 and the homologous washed red cells. This indicated that at least some of the bound C3 was bound in its active form to the platelets.

Relationship of thrombin and serum factors to platelet aggregation and retraction. Though there was evidence

that thrombin, serum factors, and platelets cooperated to facilitate clot retraction and lysis and that some of these serum factors were physically associated with the platelets, it was not known whether these factors were producing their effect directly through action on the platelet membrane or indirectly through reactions catalyzed by the surface of the platelet membrane. In other words, were thrombin and the serum factors inducing clot retraction and lysis by induction of aggregation and retraction of the platelets themselves through modification of the platelet membrane or were they acting in some other manner? The system in which serum is used to induce aggregation and retraction of washed platelets seemed to be the best system for studying this question and is described as follows.

The platelets were prepared from 18 ml of whole blood drawn into a plastic syringe containing 2 ml of a 2% solution of Na_2EDTA , pH 7.4, in 0.15 M NaCl . This preparation was centrifuged at 700 g for 10 min at room temperature. The platelet-rich supernatant was harvested, transferred to 10×100 ml siliconized tubes, and centrifuged again at 1200 g for 5 min. The supernatants were decanted and the platelet buttons were resuspended in small volumes of 0.2% Na_2EDTA -saline and pooled. These platelet pools were then brought to the volume of the test tube (15 ml) and centrifuged again at 1200 g for 2 min. The supernatants were decanted and the above procedure was repeated twice. The final button of washed platelets was reconstituted (in 0.15 M saline- Na_2EDTA , pH 7.4, 0.18 μ) to make a suspension of $1 \times 10^8/\text{mm}^3$ platelets. The yield of platelets was 30-40% of the total found in the starting material. The above procedure was carried out within 45 min of collecting the blood. 0.05 ml of this platelet suspension was placed on a siliconized slide to which 0.05 ml of fresh homologous serum alone or mixed with 0.05 ml of modifying agent had been added. The serum preparations consisted of 9 volumes of serum plus 1 volume of a 2-3 $g/100$ ml solution of CaCl_2 to give a final CaCl_2 concentration of 0.2%. The original sera were obtained from blood which was allowed to clot at 37°C for 1 hr and then refrigerated for 18 hr before harvesting. These serum preparations undoubtedly contain residual amounts of prothrombin and thrombin, a fact which is to be taken into account in the experimental procedures and discussion. A siliconized cover slip was placed over the serum-platelet preparation and was examined for platelet aggregation and retraction with the Zeiss phase microscope as described by Wright and Minot (22). The time required for aggregation and retraction to start in each of four quadrants was used as the end point. All preparations in which there was no change in platelet morphology were observed for periods up to 2 hr. If changes did occur they were graded +, and if they

did not occur they were graded —. Photographs (black and white) of the platelet preparation were taken through the 40 power objective of the phase microscope (Zeiss).

The effect of thrombin on platelet aggregation and retraction was studied by incubating 1 ml of 1×10^9 washed platelets per mm^3 (for 10 min at 22°C) with 0.1, 0.2, 0.4, 0.8, 1.0, and 2.0 U of thrombin before mixing with serum and observation as described above. Also, the effect of hirudin (10 U/ml of serum) on platelet aggregation and retraction was studied in the same manner.

The effect of cold agglutinin on platelet aggregation and retraction was studied by incubating 1 ml of 1×10^9 washed platelets per mm^3 with 0.1, 0.2, 0.4, 0.8, 1.0, and 2.0 U of thrombin together with 10 μg of cold agglutinin (for 10 min at 22°C) before mixing with serum and observation as described above.

The consumption of cold agglutinin by thrombin-treated platelets was studied by incubating 10 ml of $1 \times 10^9/\text{mm}^3$ washed platelets with and without 1–10 U of thrombin. These were washed once in 100 volumes of saline and then 0.5 ml aliquots of platelets ($1 \times 10^9/\text{mm}^3$) were added to 0.5 ml aliquots of a 1:64 dilution of high titer (1:512) cold agglutinin serum. An aliquot of cold agglutinin serum to which 1–10 U of thrombin alone was added, plus an aliquot of untreated serum were included as control samples. The mixture of platelets and sera were allowed to incubate at 4°C for 2 hr. It is important to note that the serum samples in this part of the study had no calcium added to them. The samples were then centrifuged, diluted (1:64, 1:128, 1:256, 1:512, and 1:1024) and 0.9 ml of the supernatant was added to 0.1 ml of a 1% suspension of red blood corpuscles (type O). This system was allowed to incubate in 10×75 mm agglutination tubes for 18 hr and the agglutination patterns were read with the aid of a hand lens.

Table IV shows the effect of thrombin (and cold agglutinin) on the time required for platelet aggregation and retraction to start. 0.2 U of thrombin reduced the time from 28 to 12 min. 10 μg of cold agglutinin added before the 0.2 U of thrombin reduced this time from 28 to 1 min. It was also observed that thrombin-treated platelets adsorbed more cold agglutinin from high titer cold agglutinin serum than did nonthrombin-treated platelets by at least a dilution factor of 4. However, platelets treated with thrombin alone to which no serum was added also tended to aggregate at 4°C . Therefore, it will be necessary to examine this question using radiolabeled cold agglutinin and complement components.

It should be noted that the results using thrombin have been obtained repeatedly and are in contrast to observations of Zucker and Borelli (23) and others.

TABLE IV
The Effect of Varying Concentrations of Thrombin on the Time Required for Initiation of Platelet Aggregation and Retraction

Thrombin concn	Elapsed time between addition of serum to platelets and platelet aggregation and retraction
	<i>min</i>
Serum alone	28
Serum + 0.1 U	15
Serum + 0.2 U	12
Serum + 0.8 U	8
Serum + 1.0 U	3
Serum + 2.0 U	1
Serum + 10.0 U	1

Their system consisted of platelets washed in physiologic saline and suspended at pH 7.4, whereas ours were washed in EDTA-saline at pH 7.4. It is probable that washing the platelets in the absence of a strong chelating agent may have rendered them susceptible to aggregation and retraction by thrombin alone because of Ca^{++} -dependent adherence of larger amounts of complement proteins to the membrane. Thus, in their system thrombin alone might have been sufficient to induce platelet aggregation and retraction.

The role of γM and complement components in inducing agglutination and retraction of platelets was studied further as follows.

Employing the above system the effect of cold agglutinin, anti-fibrinogen, and anti- γM as well as anti-C3 and anti-C4 on platelet aggregation and retraction was studied. In this case the reagents and platelets were mixed before addition of serum. The effect of treating serum with cobra factor (10–30 $\mu\text{g}/\text{ml}$ serum for 30 min at 37°C) or hydrazine (0.01 mole/liter at 37°C for 1 hr followed by dialysis) or potassium cyanide (equal volumes of serum and 2 M KCN incubated 18 hr at 4°C followed by dialysis) on its capacity to induce aggregation and retraction of platelets was studied. The effect of the above hydrazine-treated serum reconstituted with 40 μg of C3, 40 μg of C4, and 1 U of thrombin on platelet aggregation and retraction was also studied. The same studies were done on the above serum treated with potassium cyanide except that 40 μg of C5 was also included in the reconstitution portion of the study. Controls consisted of the chemically treated sera plus 1 U of thrombin alone, of chemically treated sera plus the complement components alone, and of thrombin plus calcium alone. The effect on platelet aggregation and retraction of C8i and C8 with and without hydrazine-treated serum was also studied.

Anti- γ M aggregated the platelets but inhibited retraction. The addition of 10 μ g of polyclonal cold agglutinin to the washed platelets also induced aggregation. The addition of 10 μ g of cold agglutinin *plus serum* and calcium induced rapid aggregation and retraction (5 ± 2 min) compared to a control consisting of serum, calcium, and platelets alone (15 ± 3 min).

Anti-C3, -C4, and -C8 added to the platelets before addition of serum failed to inhibit platelet retraction. However, treatment of the serum with either purified cobra factor (Fig. 2), hydrazine (Fig. 3), or potassium cyanide (agents which inactivate C3, C3 and C4, and C3, C4, and C5 respectively) rendered that serum incapable of mediating aggregation and retraction of platelets (Table V). Addition of purified C3 and C4 alone to hydrazine-treated serum (dialyzed, recalcified) resulted in restoration of platelet aggregation but not retraction (Fig. 4) (Table V). Addition of purified C3, C4, plus thrombin to the hydrazine-treated serum resulted in full restoration of platelet retraction (Fig. 5)

(Table V). Addition of thrombin alone to hydrazine-treated serum (dialyzed against phosphate buffer, 0.01 mole/liter, pH 7.4, and recalcified) had no effect on the platelets. Thrombin in phosphate buffer (0.01 mole/liter, pH 7.4) plus calcium aggregated the platelets but did not induce them to retract. Calcium alone also aggregated platelets but did not induce retraction. In the case of reconstitution of potassium cyanide (KCN)-treated serum; addition of C3, C4, C5, plus thrombin also resulted in full restoration of platelet aggregation and retraction. Addition of C8i plus serum produced rapid aggregation and retraction of platelets. The pH of all the above sera was maintained at 7.4 ± 0.2 . The fresh serum and serum EDTA controls to the above studies are shown in Figs. 6 and 7.

Other control studies include studies of the effect of hydrozine, potassium cyanide, and cobra factor on thrombin and plasminogen activity. These studies were done to examine the possibility that these factors, in addition to C3 and C4, might be affected by these

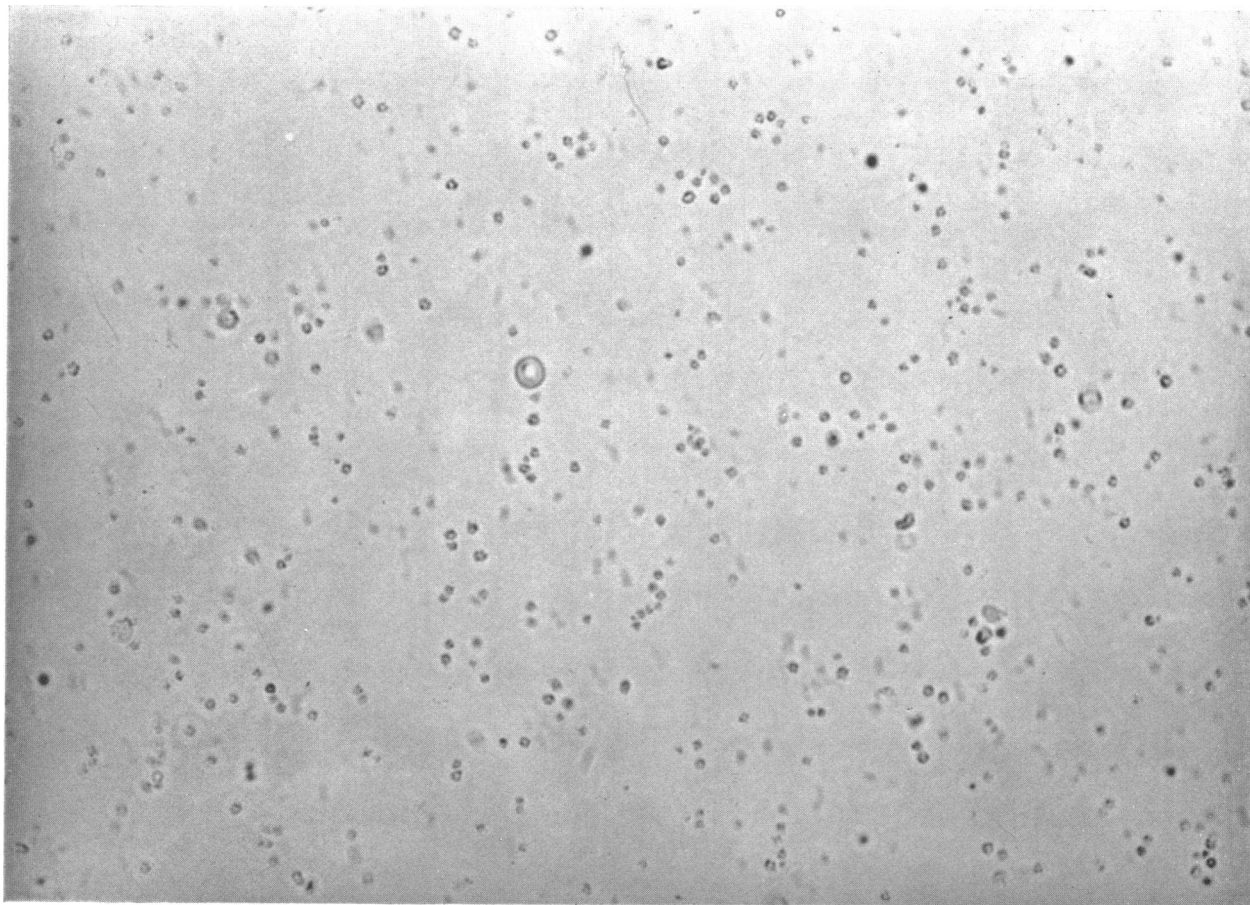


FIGURE 2 Photograph of washed platelets to which serum exposed to cobra factor (20 μ g/ml) has been added and which have been allowed to incubate at 23°C on a siliconized glass slide for 15 min. $\times 40$.

agents. In each case 10 U/ml of thrombin or 0.4 mg/ml of plasminogen were treated as described above for serum. The activity of the thrombin and plasminogen employing a 0.2% solution of fibrinogen or fibrin as substrate was assayed as described earlier. Purified plasminogen was not effected by exposure to these agents. However, purified thrombin was inactivated by hydrazine and by potassium cyanide but not by cobra factor. It should be emphasized that though hydrazine is an oxidizing agent with a broad range of activity, the conditions under which it is used in this study are quite specific for thrombin, C3, and C4. None of the other proteins being considered in this study, including plasmin inhibitors, are affected by hydrazine under these conditions.

The physical association of platelets with the fibrin clot. In order to determine whether the process of clot retraction and clot lysis was indeed associated with platelet aggregation and retraction, and in order to determine whether inhibition of clot retraction and lysis

was associated with inhibition of platelet aggregation and retraction, plasma clots were prepared from platelet-rich plasma as described previously and placed in 10% buffered formalin $\frac{1}{2}$, 1, and 2 hr after being placed in the 37°C water bath. Duplicate samples were allowed to lyse. They were allowed to fix for 3 days, and were then embedded, sectioned (10 μ), mounted, and stained as described by James et al. (17). Clots to which antibodies to γ M, C3, C4, albumin, α_2 -macroglobulin, transferrin, and γ G were added were treated in the same manner. These were viewed through the 40 power objective of a light microscope. Photographs (black and white) were then taken of these preparations.

Photomicrographs of the normal clots fixed and sectioned 2 hr after addition of thrombin showed dense masses of "fused" platelets along the periphery of a small retracted clot and only a few fibrin strands in the center. Photomicrographs of the same clot formed in presence of anti- γ M antiserum showed many small platelet aggregates and individual platelets dispersed

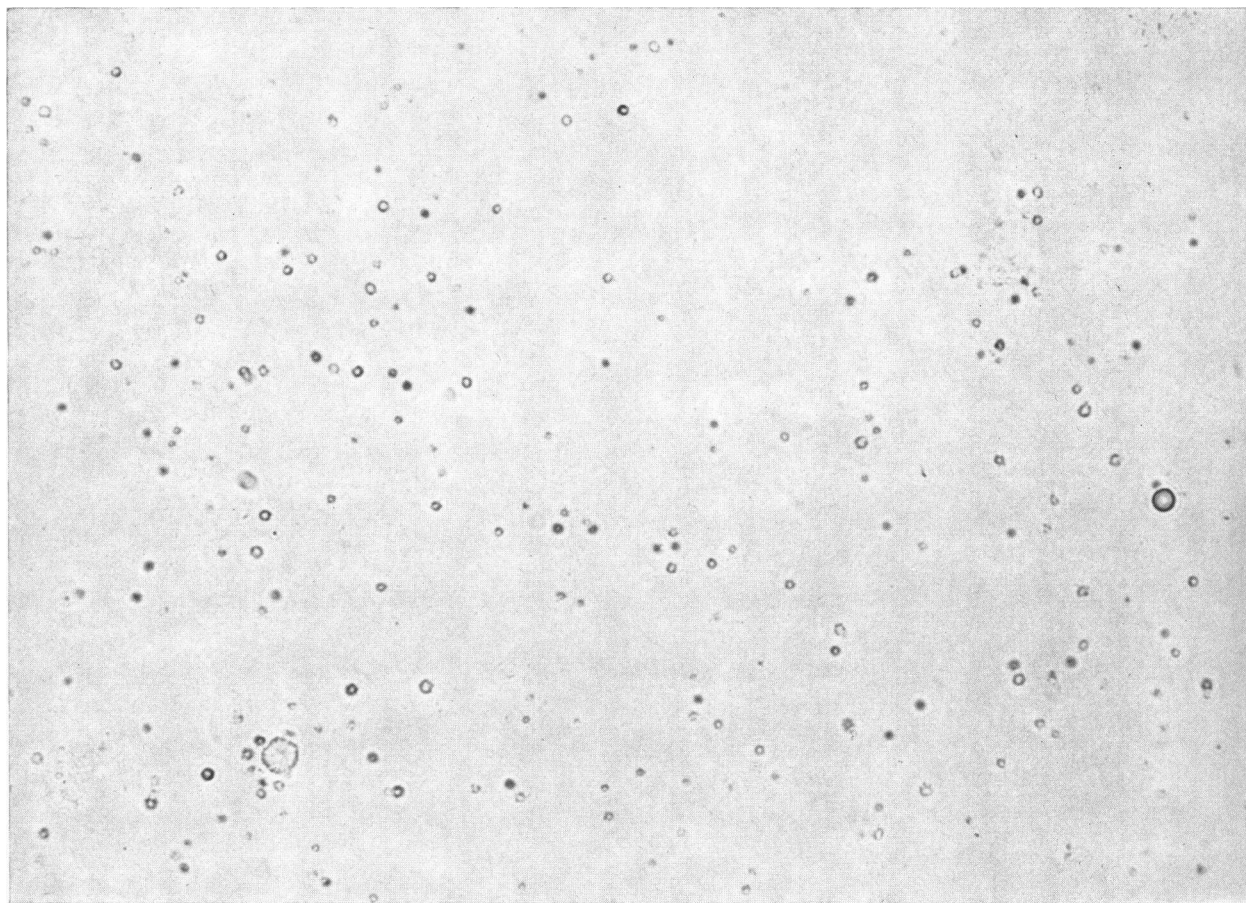


FIGURE 3 Photograph of washed platelets to which serum (exposed to 0.01 M hydrazine at 37°C for 1 hr) was added and which have been allowed to incubate at 23°C on a siliconized glass slide for 15 min. $\times 40$.

evenly throughout a field of a fine lacy fibrin network of a large unretracted clot (18).

Retraction and lysis of blood clots and the platelet proteins of samples from patients with thrombasthenia. The degree of clot retraction, of clot lysis, structure of plasma clots, and the surface proteins of platelets from patient's with Glanzman's thrombasthenia were studied with Dr. Zucker.¹ 20 ml of blood was drawn from each of six patients and three normal subjects (nonfasting). Blood samples were handled and observed for clot retraction and lysis in the same manner as described above (15). An additional 18 ml of blood as a source of platelets was handled and the platelets studied as described previously (26).

The results from these studies clearly showed that the retraction of clots from six patients with thrombasthenia was only 0-1+, whereas the retraction of clots from the three controls was 4+. The lysis time of clots from thrombasthenic patients was 6.5-24 hr (average 15 hr) compared to lysis time of 3.5-4.5 hr (average 4.0 hr) for the normal subjects. Aggregation of thrombasthenic platelets by anti- γ M and anti-fibrinogen was 0-1+ compared to 3-4+ for platelets from normal subjects (26). These studies were repeated on five different occasions on one of the thrombasthenic patients (M.M.) and on two occasions on the other two (L.W. and M.C.). Thus, there is a clear association of poor clot retraction and lysis with a deficiency of γ M and fibrinogen on the platelets of patients with thrombasthenia.

DISCUSSION

The need of platelets for lysis of dilute whole blood clots has been suggested by the lack of clot retraction and lysis which followed their removal by centrifugation. The observation that clot retraction and lysis are also inhibited following disruption of platelets by sonication suggests that the intact platelet is essential at least in the early phases of clot formation. This, in turn, suggests that the surface and(or) organization of cellular components provided by the membrane of the intact platelet is important. For, if this were not so, if it were merely the material within the platelets which was required, disruption of platelets by sonication should not have inhibited clot lysis. Further evidence of the possible importance of the platelet membrane in this system is the demonstration that a deficiency of clot

¹ One of the patients (M.M.) has been extensively studied by Dr. M. Zucker and coworkers (24). The two other patients (L.W. and M.C.) had been diagnosed and studied by Dr. Weiss (25) and the three other patients were diagnosed by Dr. Peter White of the University of Pennsylvania. The observations below correlating clot retraction and lysis with platelet proteins were done with Dr. Zucker and are reported in a recent letter (26).

TABLE V

Effect on Platelet Aggregation and Retraction of Inactivation of Serum Complement and Reconstitution of These Sera with Complement Components and Thrombin

Reagent	Platelet response on incubation with reagent	
	Aggregation	Retraction
Serum	+	+
Serum + hydrazine	-	-
Serum + hydrazine + C3 and C4	+	-
Serum + hydrazine + C3, C4, and thrombin	+	+
Serum + cobra factor	-	-
Serum + KCN*	-	-
Serum + KCN + C3 and C4	+	-
Serum + KCN + C3, C4, and C5	+	-
Serum + KCN + C3, C4, C5, and thrombin	+	+
Thrombin	-	-
Thrombin + Ca	+	-
Serum + hydrazine + thrombin	-	-

* KCN = potassium cyanide.

retraction and lysis is associated with a deficiency of fibrinogen and γ M on the membranes of platelets from thrombasthenic patients. All of the substances within platelets from these patients assayed thus far have been normal in amount and in activity (24, 25) with the exception of platelet ATPase. This was reported by Gross to be deficient in some patients with thrombasthenia (27).

Others have noted that under certain conditions platelet contents both inhibit plasmin activity and enhance plasminogen activator (SK) activity (28, 29) (i.e. platelets are known to contain plasminogen) (30). Since these studies employ specific assays for either plasmin, plasmin inhibitor, or activator activity there seems little doubt that such substances with these activities exist within the platelets. Such results do not necessarily conflict with nor support our observations in which we are monitoring the over-all contribution of the platelet within the matrix of the dilute intact blood clot. The systems are different and the results from each must be considered in the light of what activity is being measured and the assay used to measure it.

With regard to the participation of humoral factors in clot retraction and lysis, the observation that decreasing concentrations of thrombin facilitated clot retraction and lysis when added to systems clotted and run at 37°C is of interest, for, this observation shows that (a) the concentration of thrombin used on systems clotted and run at 37°C (in contrast to systems clotted at 4°C

and run at 37°C) clearly influenced the degree of clot retraction and the rate of clot lysis, and (b) the amount of thrombin added and the temperature at which the clots were allowed to form were two closely linked variables influencing the early phases of clot retraction and lysis. This observation clearly poses the possibility that the coagulation system, like other biologic systems, has the potential for initiating reactions which counteract the initial phenomena (clot formation); and that the degree of the counter phenomena or negative feedback (clot lysis) may be a function of the concentration of the initiating substance (thrombin) (see Table VI). The *biochemical* interpretation of this effect of thrombin and its relation to the conditions of pH and temperature governing the interaction of thrombin and cold agglutinin with platelet membranes will be discussed later.

Evidence suggesting the participation of a γ M protein in clot retraction and lysis rests on the complete inhibition of this phenomena by antiserum to γ M and by the

effect of adding 19S cold agglutinin to the system. Of the four γ M proteins studied (19S polyclonal cold agglutinin, 19S monoclonal cold agglutinin, 19S macroglobulin, and 19S cryoglobulin) the polyclonal 19S cold agglutinin appeared to be the active factor because this protein was the only one of the four γ M proteins which induced clot retraction and lysis of systems clotted and observed for lysis at 37°C. Ordinarily clot retraction and lysis do not occur if the clot is formed at 37°C. Usually the dilute whole blood clots will retract and lyse only if they are formed at 4°C and then observed for lysis at 37°C. However, the addition in correct amounts of 19S polyclonal cold agglutinin to clots formed at 37°C induced the same degree of retraction and lysis as that ordinarily seen in systems clotted at 4°C. Therefore, additional 19S cold agglutinin obviated the need for forming the clots at 4°C.

Evidence suggesting the participation of complement in this system rests on the inhibition of clot lysis by

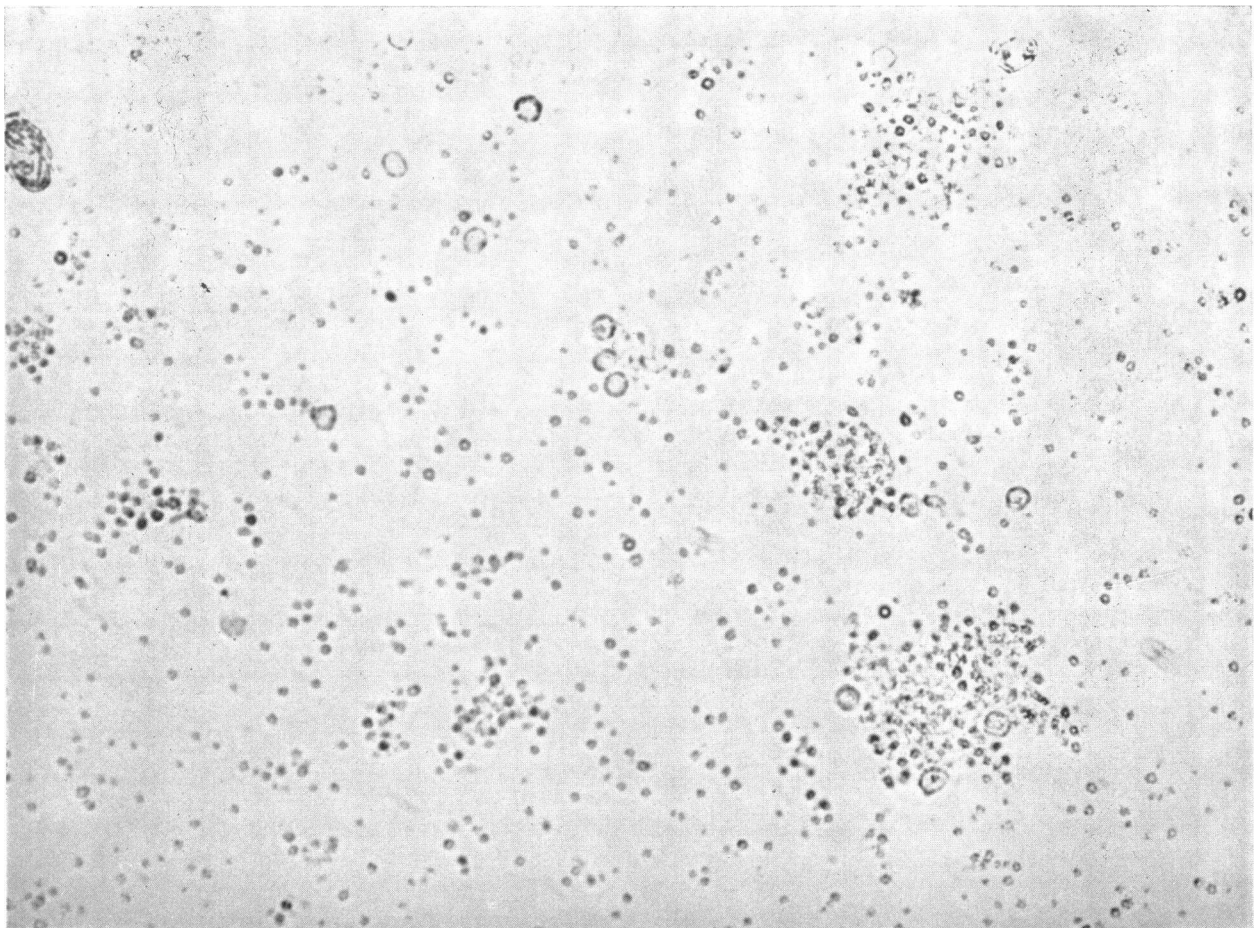


FIGURE 4 Photograph of washed platelets to which serum (exposed to 0.01 M hydrazine, dialyzed, and then reconstituted with 40 μ g/2 ml of C3 and 40 μ g of C4) was added. The platelets were then allowed to incubate at 23°C on a siliconized glass slide for 15 min. \times 40.

antibodies specifically directed against selected complement components and the fact that this inhibition was not observed in those assays in which the homologous antigens were included. Also the evidence that antibodies directed against other serum proteins (albumin, α_2 -macroglobulin, transferrin, and γ G globulin) failed to inhibit clot lysis, strengthened the thesis that the inhibition by the antisera directed against the complement components was specific. The facts that (a) the pH optimum of clot lysis was 6.5 and not 7.4 and (b) lysis was inhibited by cobra factor and restored by adding C3 are also consistent with the interpretation that complement is involved in this process. The later observation on the effect of cobra factor and C3 supports the observations made using antisera. One of the weaknesses of studies employing antisera lies in the possibility of antibodies to trace contaminants (less than 5%) may inactivate active principle which has been mistaken to be the protein in the highest concentration. Another

difficulty in studies employing antisera is that the function of the system may be inhibited by binding of antisera to proteins which are *nonspecifically* adsorbed to surfaces on which the reactions are occurring (i.e. fibrin strands, platelet membranes). However, the fact that an agent such as cobra factor can also produce inhibition of clot retraction and lysis tends to negate these possibilities.

In remarking on the limits of these experiments it must be noted that control samples to which 19S cold agglutinin, or C3 or C4 alone were added did not accelerate retraction or lysis as might be expected. Only the addition of platelets and C8i accelerated both clot retraction and lysis, and the addition of plasminogen accelerated clot lysis. If one assumes that the cold agglutinin and early components of complement act through binding to a limited number of sites on a surface (e.g. the platelet membrane or fibrin strand), then the fact that additional cold agglutinin, or C3 or C4

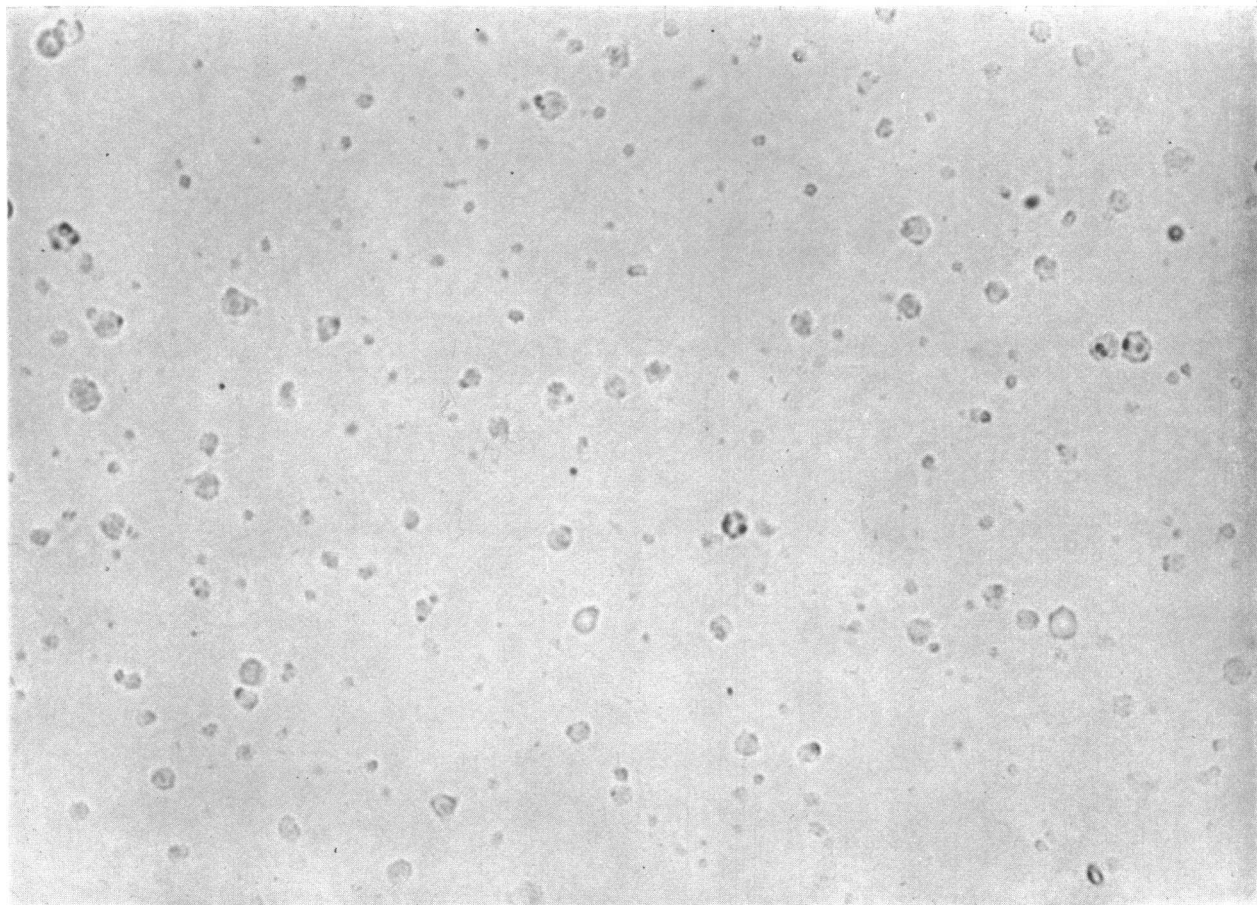


FIGURE 5 Photograph of washed platelets to which serum (exposed to 0.01 M hydrazine, dialyzed, and then reconstituted with 40 μ g/2 ml of C3 and 40 μ g of C4 plus 10 μ g of thrombin) was added. The platelets were then allowed to incubate at 23°C on a siliconized glass slide for 15 min. \times 40.

added at 4°C did not accelerate lysis might be explained by assuming that only a restricted number of appropriate or specific sites on a surface are available at 4°C.

The action of C8i on the clot retraction and lysis system (the nonhemolytic form of C8) then came under examination in a preliminary study. Since it was found that C8i markedly accelerated clot lysis, the question was raised of whether it was an activator of plasminogen or had a capacity to hydrolyze fibrin itself. From the data obtained from assays done using a purified fibrin substrate, we concluded that C8i or its equivalent might be a component which activates plasminogen. Why only the hemolytically inactive form of C8 and not C8 itself is active on plasminogen is not known. Therefore, in contrast to the other reactants described above, we cannot argue conclusively that C8 is an essential component in this phenomena, we can only suggest that some form of C8 may be involved.

Finally, the observation that either one of two sets of conditions (pH 7.4, 4°C, or pH 6.5, 37°C) would permit optimal clot retraction and lysis is of great interest and is consistent with the observation that optimal binding of cold agglutinin occurs under either one of these two conditions. As stated previously, the relation of this phenomena to thrombin and the conditions of pH and temperature governing the interaction of thrombin and cold agglutinin with platelet membranes will be discussed later. These last observations complete the qualitative identification and study of some of the components or factors thought to be involved in facilitation of clot retraction and lysis.

Regarding the conditions under which clot lysis studies were done, it should be noted that the ionic strength and pH of the buffer used is optimal for binding and activation of C1, C3, and C5, 6, 7. It should also be noted that this low ionic strength does no harm to platelet or red corpuscles because of the contribution to the osmotic

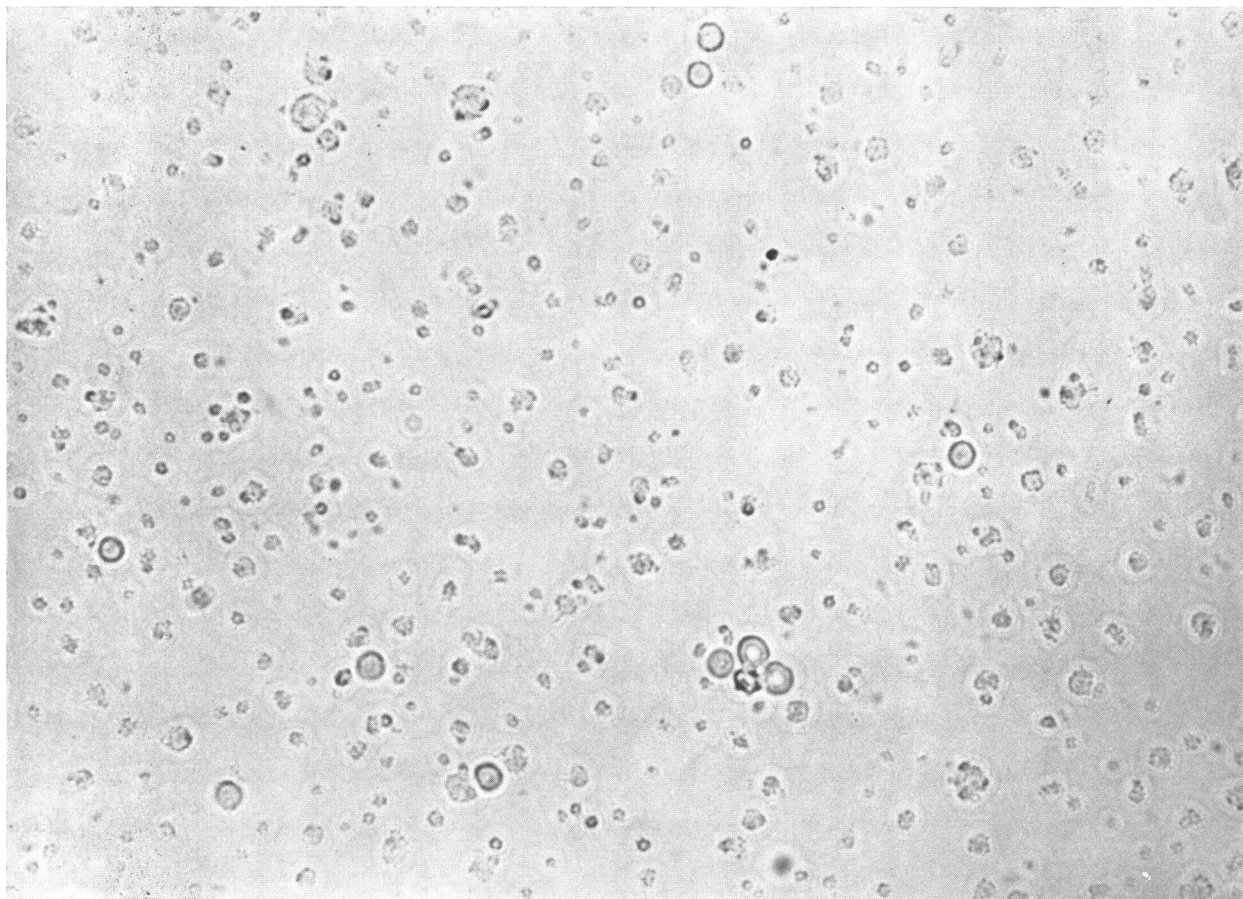


FIGURE 6 Photograph of washed platelets to which serum was added and which have been allowed to incubate at 23°C on a siliconized glass slide for 15 min. Each of the masses shown represent islands of 30-40 platelets which have aggregated, and retracted to one-fifth their original size. $\times 40$.

gradient made by the dilute plasma salts and proteins. However, the concentration of both free calcium (5×10^{-4} mole/liter) and of available cold agglutinins is below that required for optimal complement activity.

It was now necessary to establish whether platelets and those serum factors shown to be necessary for clot retraction and lysis were physically associated. More specifically the first question was whether fibrinogen, γ M globulin, and complement components C1-4 were physically associated with platelets. The demonstration that platelets (washed four times) could be specifically agglutinated by antibodies directed against fibrinogen, γ M, C3, or C4 gave evidence that this was so. The demonstration that C3 adsorbed onto the platelets could mediate immune adherence with indicator red corpuscles suggested that at least some of C3 bound to the platelet membrane was bound specifically and was activated under these conditions. Further evidence supporting the specificity of binding of these factors was provided by the failure to agglutinate these washed platelets with antibodies to some other serum proteins and by the fact

that where agglutination was produced by a given antibody, it could be prevented by addition of the homologous antigen (i.e. 19S cold agglutinin, C3, C4). These series of experiments therefore provided evidence that fibrinogen, γ M globulin, and complement components are physically associated with platelets under certain conditions. These observations, together with the functional evidence given previously, raised the question of whether the platelets and serum proteins actually functioned as a unit in promoting clot retraction and lysis. This question was advanced further by (a) the observation that the platelet membranes of patients with thrombasthenia have markedly reduced amounts of fibrinogen and γ M, and (b) the fact that this defect was associated with the failure of the dilute whole blood clots of these patients to retract or lyse normally and by a failure of their platelets to aggregate and retract upon exposure to serum. Though this association cannot be interpreted as a causal one, it was conceivable that the platelets of thrombasthenic patients fail to function because they do not provide a surface to which a γ M globulin can

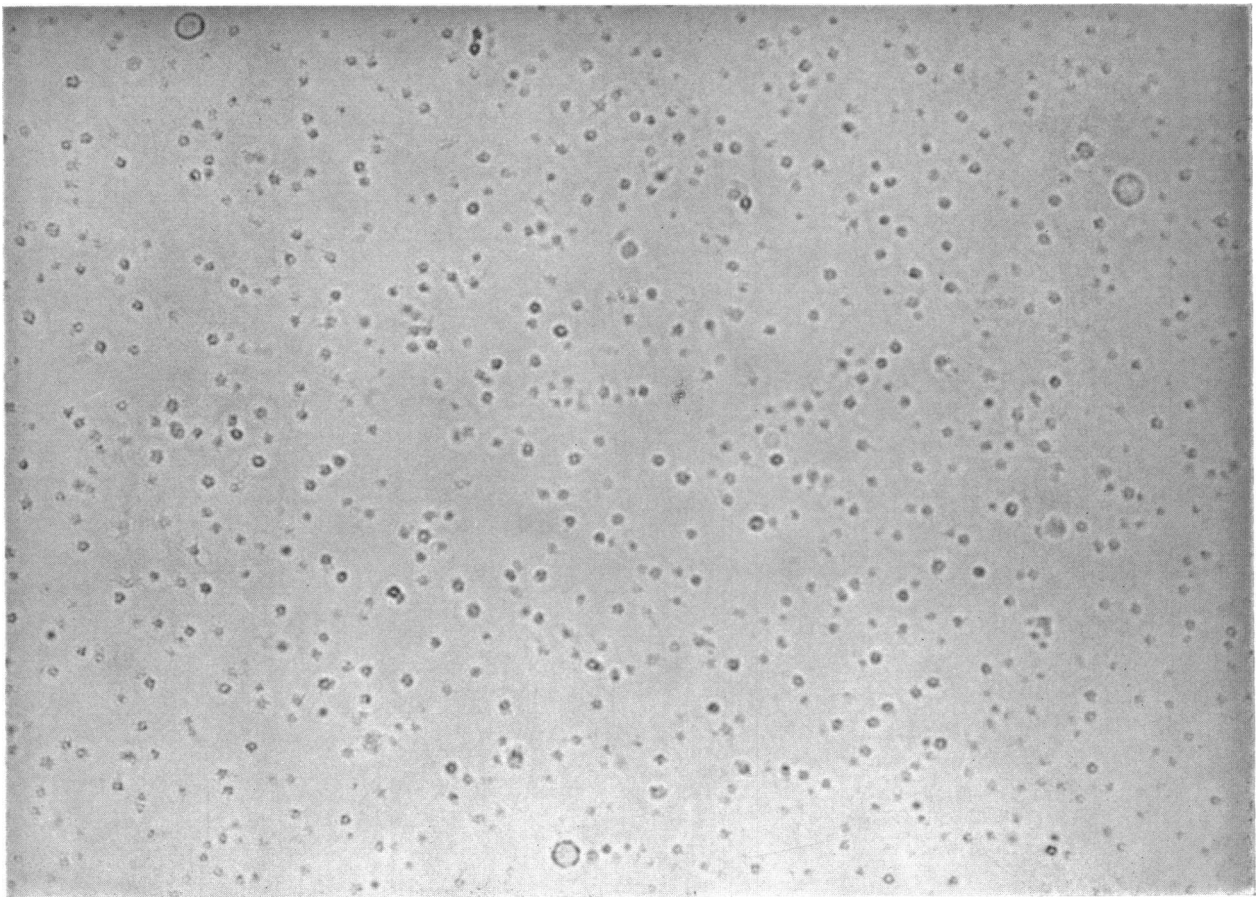


FIGURE 7 Photograph of washed platelets to which serum and EDTA has been added and which have been allowed to incubate at 23°C on a siliconized glass slide for 15 min. $\times 40$.

become attached at 4°C and (or) pH 6.5 (possibly to thrombin-modified platelet fibrinogen or to the I antigen) (19, 31). Such a failure to bind γ M globulin might be followed in turn by a failure to bind and activate C1 (32) at 4°C followed by activation and binding of the other complement components to the platelet membrane at 37°C in a manner analogous to that described for complement-mediated hemolysis of red blood corpuscles.

If one accepts this interpretation of the abnormality of the thrombasthenic platelets, and if one accepts the evidence that platelets, thrombin, γ M globulin (cold agglutinin), and serum factors are physically associated with the platelets, it seems reasonable to postulate that clot retraction and lysis may result from functional interaction between these serum factors and the platelets. If this reasoning is followed, at least two questions arise. (a) How do the serum factors get adsorbed onto the platelet membrane, and (b) what is their function once they are there and which factors are involved?

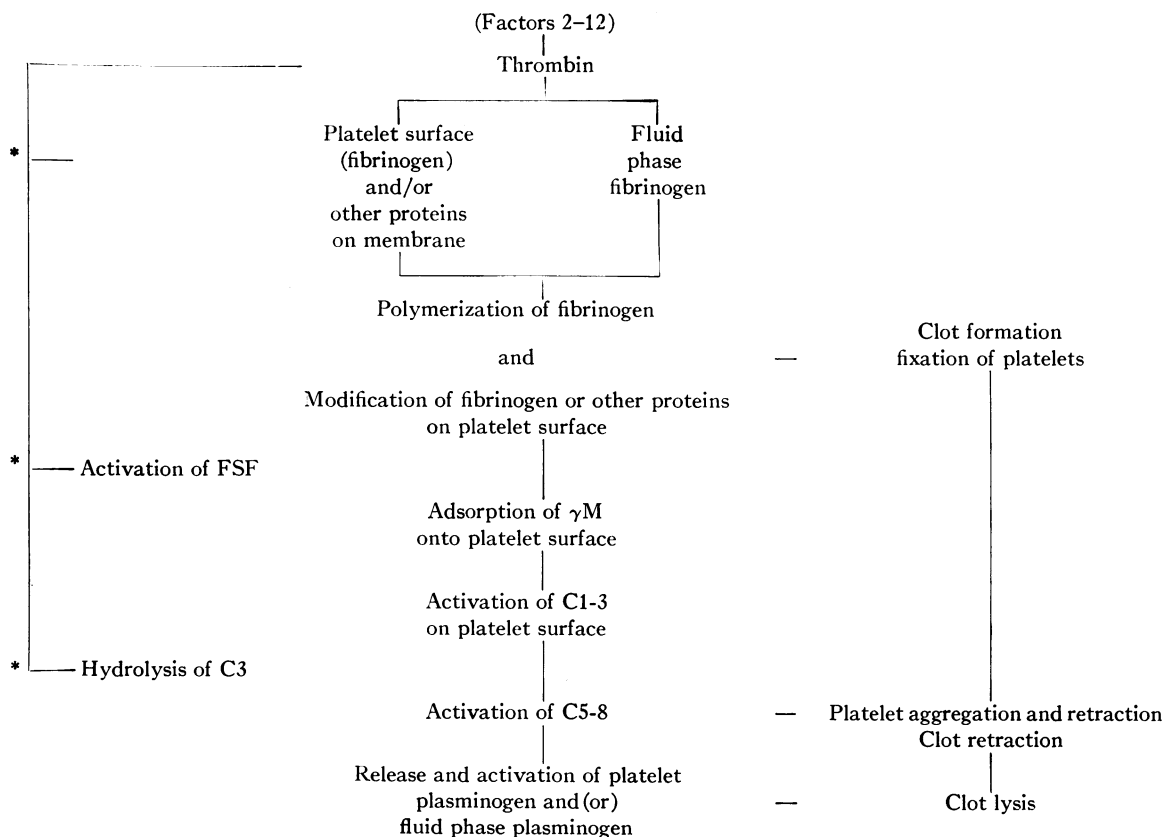
Do they mediate reactions limited to the membrane surface or do they modify the membrane?

Looking at serum-mediated retraction and aggregation of platelets it was found that either hirudin or anti- γ M antibody added to the fresh washed platelets inhibited their aggregation and retraction upon addition of serum. This suggested that the thrombin and γ M (cold agglutinin) present in serum might play a role in the induction of platelet aggregation and retraction. The fact that addition of more thrombin and (or) γ M globulin (cold agglutinin) to the washed platelets markedly enhanced the rate of serum-induced platelet retraction and fusion, strengthened the interpretation that both thrombin and γ M globulin (cold agglutinin) were operative in this phenomena just as they were in the retraction and lysis of dilute whole blood clots.

It was then hypothesized that the thrombin modified the membrane of the washed platelet such that it would adsorb more than the *usual* amount of γ M globulin (cold agglutinin). An indirect test of this hypothesis

TABLE VI

Proposed Sequence of Events in Retraction and Lysis of Dilute Whole Blood Clots



* Points at which high concentrations of thrombin might conceivably modify the above system to favor a state of coagulation, without clot lysis.

was carried out by determining the amount of cold agglutinin adsorbed from a high titer heat-inactivated cold agglutinin serum by nonthrombin-treated and by thrombin-treated platelets. The fact that more cold agglutinin was adsorbed by the thrombin-treated platelets than by the nonthrombin-treated platelets suggested that the thrombin in serum or added thrombin might indeed modify the platelet membrane such that additional amounts of cold agglutinin were adsorbed onto the platelets.

Given the observation on retraction and lysis of clots, and the evidence that adsorbed cold agglutinin is capable of binding and activating complement on red cell membranes (32), it was but a small step to hypothesize that platelets with adsorbed cold agglutinin recruited the complement of serum to mediate the intermediate steps of platelet aggregation and retraction. The fact that inactivation of complement components of serum by cobra factor, or hydrazine, or potassium cyanide also inhibited serum-induced aggregation and retraction of platelets strengthened this hypothesis. Its validity was more firmly established by the fact that the capacity of serum to support this phenomenon was restored by addition of purified thrombin, C3, C4, and C5. The effect of C8i and serum on platelet membranes, though not conclusive, suggests that the terminal complement components also may be operative in serum-induced aggregation and retraction of platelets. Thus, the evidence suggests that thrombin, γ M globulin (cold agglutinin), C1-5, and possibly C8 may mediate the aggregation and retraction of platelets just as they influence the retraction and lysis of dilute whole blood clots.

Therefore, since platelet aggregation and retraction is said to lead to clot retraction (33), and clot retraction is invariably associated with clot lysis, and since clot retraction and lysis are inhibited by removal of platelets and inactivation of the same serum factors as those required for serum-mediated platelet aggregation and retraction, we have concluded that (Table VI) (a) thrombin in addition to catalyzing clot formation also modifies the platelet membrane such that γ M globulin (cold agglutinin) and complement act (more readily) on the platelet membrane leading to (b) platelet aggregation and retraction together with retraction of the clot and (c) activation of plasminogen either on the surface of the platelet by C8i and (or) by release of platelet activators of plasminogen (Table VI).

However, in considering this conclusion the following four questions must be examined.

1. On one hand, are platelet aggregation and retraction and clot retraction (which is clearly thrombin, γ M, fibrinogen, and complement dependent) necessary steps leading to clot lysis? On the other hand, are platelet aggregation-retraction and clot lysis two independent

phenomena which both share components of a common effector system? The fact that clot retraction invariably precedes clot lysis (in this system) and that failure of clot retraction invariably is associated with prolonged clot lysis suggests that platelet aggregation and retraction and clot retraction are essential steps leading to clot lysis (Table VI). Thus, it might be reasoned that thrombin, fibrinogen, γ M, and complement facilitate clot lysis through their direct mediation of platelet aggregation and retraction and clot retraction. However, this cannot be established directly until the platelet-effector system is constructed from platelets and purified components.

2. How does thrombin initiate the γ M-complement interaction with platelets? Does it act by modifying the platelet membrane or platelet fibrinogen? The facts that (a) thrombin action on the platelet membrane leads to increased uptake of cold agglutinin, and that (b) the 4°C temperature step required for initiation of normal clot retraction and lysis can be obviated either by adding a low concentration of thrombin, by decreasing the pH to 6.5 (optimal for cold agglutinin binding), or by adding more cold agglutinin are of interest. They suggest that the concentration of thrombin and cold agglutinin, and the conditions of temperature and pH within the intact clot may be closely related, in governing their reaction with a common substrate on the platelet membrane and (or) the fibrin strand. With regard to this reaction and assuming that the initial reactants are restricted to thrombin, platelet fibrinogen, and γ M globulin, the following possibilities for a substrate arise. (a) Thrombin could alter the fibrinogen on the platelet membrane such that at 4°C or pH 6.5 cold agglutinin could associate with the modified fibrinogen (fibrin) or the I antigen; (b) thrombin could modify cold agglutinin such that it could bind more readily to the platelet membrane or fibrinogen at 4°C; (c) the cold agglutinin could bind to thrombin at 4°C and modify its activity such that clot lysis was enhanced in some manner; or (d) thrombin could initiate a series of reactions at the platelet membrane through hydrolysis of proteins other than fibrinogen. For instance, hydrolysis of C3 by thrombin has been demonstrated in our laboratories to be highly efficient. The second of the four possibilities listed is of great interest to us and may warrant more serious consideration, for repeated preliminary studies show that polyclonal cold agglutinin and fibrinogen in the presence of thrombin form a complex which will not polymerize, whereas no complex is formed between cold agglutinin and fibrinogen in the absence of thrombin.

The answer to this question will rest on further studies of the thrombin-fibrinogen-cold agglutinin reaction and on the construction of a platelet-effector system model from platelets and purified components.

3. How do C8i or platelet contents, or both, activate plasminogen? Again the attempt to answer this question awaits construction of a platelet-effector system model from platelets and purified components.

4. Assuming that thrombin may prepare the platelet membrane in some way such that *more* γ M (cold agglutinin) and complement are adsorbed and are activated, how does thrombin affect the above system such that a low concentration of thrombin favors clot retraction and rapid lysis and a high concentration favor poor retraction and slow lysis? One possibility is that a high concentration of thrombin interferes with the effector system at some point other than at the membrane. Thrombin will hydrolyze C3. Therefore, it is conceivable that a higher concentration of thrombin not only would initiate polymerization but also hydrolyze C3 or one of the other factors thus interrupting the series of reactions leading to retraction and lysis (Table VI). However, this cannot be established until the platelet-effector system is constructed from purified components and the effect of thrombin tested.

How ever these points are eventually answered, we believe that the systems and findings described above, though perhaps artifactual, are worthy of further study. We believe that they demonstrate how the coagulation and lytic enzyme systems and the immunoglobulin-complement systems cooperate in an ordered sequence of reactions in the unique milieu provided by the fibrin-platelet surface. We also believe that these findings allow for the first time an estimate to be made of the intermediate steps involved which link the events associated with the generation of thrombin (coagulation factors 12-2), clot retraction (release of thrombasthenin), and clot lysis.

These findings and the model derived therefrom may also facilitate the definition of those factors which are absent or functioning abnormally in certain diseases of the coagulation system such as the platelet disorders in thrombasthenia, and paroxysmal nocturnal hemoglobinuria, and the immunocoagulation disorders in systemic lupus erythematosus and disseminated intravascular coagulation. Further, we hope that models such as this may provide rational constructs from which the immunopathologic aspects of coagulation and diseases of vessel walls can be examined and interpreted.

ACKNOWLEDGMENTS

We thank Jorene Moore, Ilona Staprans, Rhoda Sheena, Gloria Heym, Joanne Beisswenger, and Linda Pomenti for their technical assistance over the 10 yr period that this work was developing. We also wish to thank Doctors A. F. Bickford, H. Fudenberg, E. Becker, Howard Rawnsley, and S. Sherry for their advice and help and Mrs. E. Corbelli for her inspiration.

2084 F. B. Taylor, Jr., and H. J. Müller-Eberhard

This work was supported by U. S. Public Health Service Grants HE-25-155, AI-00319, and HE 10-907 and an American Heart Association Grant.

REFERENCES

1. Cohen, S. I., and R. Warren. 1961. Fibrinolysis. *N. Engl. J. Med.* 264: 79.
2. Mehrotra, T. N., and P. A. Charlwood. 1960. Physico-chemical characterization of the cold auto-antibodies of acquired haemolytic anaemia. *Immunology.* 3: 254.
3. Haines, A. L., and I. H. Lepow. 1964. Studies on human C'1-esterase. I. Purification and enzymatic properties. *J. Immunol.* 92: 456.
4. Müller-Eberhard, H. J., U. Nilsson, and T. Aronsson. 1960. Isolation and characterization of two B₁-glycoproteins of human serum. *J. Exp. Med.* 111: 201.
5. Müller-Eberhard, H. J., and C. E. Biro. 1963. Isolation and description of the fourth component of human complement. *J. Exp. Med.* 118: 447.
6. Nilsson, U. R., and H. J. Müller-Eberhard. 1965. Isolation of B_{1F}-globulin from human serum and its characterization as the fifth component of complement. *J. Exp. Med.* 122: 277.
7. Kabat, E. A., and M. M. Mayer. 1961. Electrophoresis and immunoelectrophoresis. In *Experimental Immunochimistry*. Charles C Thomas, Springfield, Ill. 2nd edition. 622.
8. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* 121: 404.
9. Biggs, R., and R. G. Macfarlane. 1962. *Human Blood Coagulation and its Disorders*. F. A. Davis Company, Philadelphia. 3rd edition. 390.
10. Norman, P. S. 1957. Studies of the plasmin system. I. Measurement of human and animal plasminogen. Measurement of an activator in human serum. *J. Exp. Med.* 106: 423.
11. Taylor, F. B., Jr., and I. Staprans. 1966. Chromatography, physical characteristic, and disulfide bonds of euglobulin plasminogen. *Arch. Biochem. Biophys.* 114: 38.
12. Taylor, F. B., Jr., and J. Botts. 1968. Purification and characterization of streptokinase with studies of streptokinase activation of plasminogen. *Biochemistry.* 7: 232.
13. Brown, M. E., and F. Rothstein. 1967. Fibrinogen from human plasma: preparation by precipitation with heavy-metal coordination complex. *Science (Washington).* 155: 1017.
14. Kabat, E. A., and M. M. Mayer. 1961. Gamma (gamma₂) globulin. In *Experimental Immunochimistry*. Charles C Thomas, Springfield, Ill. 2nd edition. 760.
15. Fearnley, G. R., G. Balmforth, and E. Fearnley. 1957. Evidence of diurnal fibrinolytic rhythm; with a simple method of measuring natural fibrinolysis. *Clin. Sci.* 16: 645.
16. Bickford, A. F., and F. B. Taylor. 1963. Role of platelets in dissolution of blood clots. *Nature (London).* 199: 610.
17. James, T. N., S. A. Johnson, R. W. Monto, G. Diab, and J. Caldwell. 1962. Histology of platelet-plasma clots from normal subjects and patients with abnormal coagulation. *Blood.* 19: 751.
18. Taylor, F. B., and H. J. Müller-Eberhard. 1967. Factors influencing lysis of whole blood clots. *Nature (London).* 216: 1023.

19. Dacie, J. V. 1950. The presence of cold haemolysins in sera containing cold haemagglutinins. *J. Pathol. Bacteriol.* **62**: 241.
20. Müller-Eberhard, H. J., A. P. Dalmasso, and M. A. Calcott. 1966. The reaction mechanism of β_{10} -globulin (C'3) in immune hemolysis. *J. Exp. Med.* **123**: 33.
21. Nishioka, K. 1963. Measurements of complement by agglutination of human erythrocytes reacting in immune-adherence. *J. Immunol.* **90**: 86.
22. Wright, J. H., and G. R. Minot. 1917. The viscous metamorphosis of the blood platelets. *J. Exp. Med.* **26**: 395.
23. Zucker, M. B., and J. Borrelli. 1961. Changes in platelet adenosine triphosphate concentration and phosphate distribution during viscous metamorphosis and clot retraction. In Henry Ford Hospital International Symposium Blood Platelets. S. A. Johnson, editor. Little, Brown & Co., Boston. 383-391.
24. Zucker, M. B., J. H. Pert, and M. W. Hilgartner. 1966. Platelet function in a patient with thrombasthenia. *Blood.* **28**: 524.
25. Weiss, H. J. 1967. Platelet aggregation, adhesion and adenosine diphosphate release in thrombopathia (platelet factor 3 deficiency). A comparison with Glanzmann's thrombasthenia and von Willebrand's disease. *Amer. J. Med.* **43**: 570.
26. Taylor, F. B., and M. B. Zucker. 1969. Prolonged clot lysis time and absence of platelet γ M-globulin in patients with thrombasthenia. *Nature (London)*. **222**: 99.
27. Gross, R. 1967. Platelet enzymes. *Thromb. Diath. Haemorrhag.* **26** Suppl: 143.
28. Hougie, C., and F. Ayers. 1960. Lipaemia and fibrinolytic activity potentiality. *Lancet.* **1**: 186.
29. Holemans, R., and R. Gross. 1961. Influence of blood platelets on fibrinolysis. *Thromb. Diath. Haemorrh.* **6**: 196.
30. Nachman, R. L. 1965. Immunologic studies of platelet protein. *Blood.* **25**: 703.
31. Lalezari, P., and G. Bernard. 1967. Cold reacting anti-leukocyte and platelet antibody in infectious mononucleosis. *J. Clin. Invest.* **46**: 1081. (Abstr.)
32. Rosse, W. F., T. Borsos, and H. J. Rapp. 1967. Reaction of cold agglutinating antibodies with I⁺ and I⁻ red blood corpuscles. *J. Clin. Invest.* **46**: 1111. (Abstr.)
33. Zucker, M. B., and J. Borrelli. 1958. Some effects of divalent cations on the clotting mechanism and the platelets of EDTA blood. *J. Appl. Physiol.* **12**: 453.