Biochemical and Biophysical Aspects of Human Platelet Adhesion to Collagen Fibers

BRUCE LYMAN, LAWRENCE ROSENBERG, and SIMON KARPATKIN

From the Department of Medicine, New York University Medical School, New York 10016

ABSTRACT A method has been developed for measuring the adhesion of platelets to purified collagen fibers obtained from bovine tendon. This method differs from others in that: (*a*) platelet adhesion is measured in the absence of platelet aggregation; (*b*) platelet-rich plasma collected in ACD (acid citrate dextrose) or EDTA, or washed platelets can be employed; (*c*) adherent platelets are enumerated directly; (*d*) erythrocytes and leukocytes do not adhere.

Washed platelets suspended in human Ringer solution exhibit negligible adhesion (at the platelet concentrations employed) in contrast to washed platelets suspended in plasma. Addition of purified human fibrinogen (95% clottable, 2–4 mg/ml) to human Ringer solution completely restores the ability of washed platelets to adhere to collagen fibers. Albumin (fatty acid free, 50 mg/ml) is also capable of restoring adhesion. Albumin and seven other proteins at concentrations of 5–10 mg/ml, with varying molecular weights, isoelectric points, and frictional coefficients are incapable of supporting the adhesion of washed platelets. The proteins tested were human globulin, hexokinase, hemoglobin, cytochrome-C, insulin, thyroglobulin, and muramidase. Platelet adhesion is proportional to both platelet concentration and fibrinogen concentration, but is independent of temperature or glycogen stores.

Modification of fibrinogen by acylation of amino groups or removal of sialic acid has no effect on its ability to support platelet adhesion. Degradation of fibrinogen with purified plasmin results in decreased support of platelet adhesion. This accompanied formation of early breakdown products with clottability ranging from 84–0%. Formation of fibrinogen degradation products was monitored by SDS-polyacrylamide gel electrophoresis of the corresponding fibrins after reduction of disulfide bonds (a method capable of distinguishing α-, β- and gamma-chains). Decreased support of platelet adhesion is associated with the disappearance of intact α- chains and early modification of the β-chains.

Purified proteinpolysaccharide macromolecules obtained from bovine nasal and humeral cartilage, and from nucleosus pulposus are as effective as fibrinogen on a weight basis and ten to thirty times more effective on a molar basis in supporting platelet adhesion. The purified mucopolysaccharide side chains: chondroitin-4-sulfate, chondroitin-6-sulfate, and keratan-sulfate are incapable of supporting platelet adhesion.

INTRODUCTION

Although considerable information has been accumulated regarding the biochemistry and physiology of platelet-plug formation (1–7), little is known about initiation of platelet plug formation, namely platelet adhesion. Early work, employing light microscopy, (8–10) had suggested that platelets adhered to traumatized mesenteric fibers which were of "collagen" nature. More recent electron miscroscopic investigation has implicated the vascular basement membrane as the probable site of platelet adhesion (11–13). Several types of epithelial and endothelial basement membranes have been characterized (14–16). These have been shown to be related to (but not identical with) the collagen class of proteins. Since various collagen preparations have been shown to share the property of inducing platelet aggregation (10, 17–21), or providing a surface to which platelets adhere (19, 21–23), considerable interest has been generated in this "physiologic" interaction of platelets with collagen.

Paradoxically, most available techniques for the measurement of the adhesive properties of platelets have employed glass as the surface to which adhesion was measured (24–31). Results obtained with various tests from different laboratories are often inconsistent (28), as well as unpredictable (27–31). Emphasis has been

This work was presented at the 13th Annual Meeting of the American Society of Hematology at San Juan, Puerto Rico, 8 December 1970.

Received for publication 21 January 1971 and in revised form 17 February 1971.

placed on methods in which platelets are counted before and after exposure of platelet suspensions or whole blood to glass surfaces. Under these conditions, aggregation often occurs (24, 26, 27), and the test is better referred to as a platelet retention test.

The purpose of this investigation was twofold: 1. To develop a method for the measurement of platelet adhesion which would (*a*) employ purified collagen as the surface to which platelets adhere; (*b*) quantitate platelet adhesion directly; (*c*) provide an assay for platelet adhesion uncomplicated by superimposed aggregation; (*d*) permit the use of washed human platelets in the system so that various suspending media could be evaluated. 2. To study some of the biochemical and biophysical aspects of platelet adhesion.

METHODS

Collagen surface preparation. Bovine tendon collagen, 200 mg, was homogenized in 100 ml of 0.03 N HCl for 20 min at 0°C in a Sorvall Homogenizer. The homogenate was centrifuged at 3000 *g* for 30 min and a clear supernatant obtained. This supernatant was extensively dialyzed against isotonic saline at room temperature until a fine flocculant precipitate of collagen fibers was formed. The collagen fiber suspension was concentrated by sedimentation to approximately 10 ml by standing overnight at 4°C. 2 ml of this suspension was pipetted on to 22 × 22 mm plastic cover slips and allowed to dry at room temperature. The collagen-coated surface thus formed was rinsed free of salt crystals with distilled water. A scissors was employed to trim 2 mm from the edges of the collagen-coated cover slips. The cover slips were then divided in two, to obtain surfaces measuring 9 × 18 mm.

Adhesion assay procedure. The long edge of a collagen-coated plastic cover slip was clasped and held by a slit made through the end of a plastic "drinking straw." The collagen coated surface was immersed in isotonic saline at 37°C for 1 hr. Each substance, whose ability to support adhesion was to be measured, was dissolved in human Ringer solution-0.1 mM EDTA, adjusted to pH 7.1 (if necessary) and centrifuged at 35,000 *g* for 15 minutes. Five ml were then pipetted into each of four wide-mouthed plastic scintillation vials which were then incubated at 37° for 10 minutes. Washed human platelets were then added to vial number one by the addition of 0.5 ml of a 5% (volume) suspension of platelets in human Ringer-0.1 mM EDTA. All vials were then kept for an additional 15 min at 37°C. A collagen-coated coverslip, held by a plastic drinking straw, was then removed from the isotonic saline solution, blotted briefly on its edge to remove excess fluid, immediately dipped into vial number one, and gently agitated back and forth for 1 min. (Longer durations of incubation, for up to 15 min, did not result in increased adhesion.) It was removed from vial number 1 and held horizontally (collagen side up) by its edges. The straw was removed, blotted thoroughly, and reattached to the collagen surface at its original location. The collagen-coated cover slip was then dipped gently into the first rinse vial, vial number 2, and slowly agitated back and forth, three times. This procedure was repeated for rinse vials numbers 3 and 4. After the final rinse, the cover slip was placed on a glass microscope slide, collagen side up. One drop of solution from vial number 4 was placed on the collagen surface and the slide observed under phase optics using a 40X objective. Platelets remaining adherent to the collagen surface were enumerated with the help of a grid which outlined a surface area of 0.175 × 0.120 mm on the collagen surface. Twenty grid areas were counted in the central portion of the collagen surface toward the end opposite to which the plastic straw had been attached. Cover slips were incubated in duplicate and adhesion was expressed as the average number of platelets per grid.

Preparation of purified human fibrinogen. Platelet-poor plasma was obtained from whole blood anticoagulated with ACD (acid citrate dextrose) or EDTA. Epsilon-amino caproic acid (EACA)[1] was added to the platelet-poor plasma to a final concentration of 0.2 M. The plasma was then stored frozen at −20°C until ready for use. Fibrinogen was precipitated by glycine (32) and dissolved in a minimum volume of 55 mM sodium citrate buffer, pH 7.4 containing 0.2 M EACA (buffer-EACA), and stored frozen at −20°C until ready for use. For each adhesion experiment, human fibrinogen was prepared fresh, by either dialyzing the stored solution in buffer against human Ringer-0.1 mM EDTA (1:100) at 4°C overnight, or by reprecipitating the fibrinogen with glycine and redissolving the fibrinogen in human Ringer-0.1 mM EDTA. Fibrinogen concentration was determined by optical density readings at 280 mμ assuming an absorbance of 1% = 15.0 (33). The per cent clottability was determined from the initial optical density and from the optical density of the clot liquor (34), after addition of 10 U/ml of bovine thrombin.

Preparation of fibrinogen degradation products. Human fibrinogen was dissolved in human Ringer-0.1 mM EDTA as described above, and a portion immediately brought to 0.2 M in EACA and stored frozen. A 2nd portion (6–10 mg/ml) was treated with human plasmin. At intervals, portions were removed, brought to 0.2 M EACA, and frozen at −20°C. These preparations were tested for clottability as described above, and tested for thrombin time and antithrombin activity as described below. Clots from these preparations were reduced and electrophoresed on SDS (sodium dodecyl sulphate) polyacrylamide gel as described previously (34).

Preparation of neuraminidase-treated fibrinogen. Purified human fibrinogen (5–10 mg/ml), was incubated in the presence of buffer-EACA, pH 5 with 6.7 μg neuraminidase per mg fibrinogen for 1 hr at 37°C. Release of free sialic acid was assayed by a modification of the thiobarbituric acid method (35) and expressed as N-acetylneuraminic acid, as determined from a standard curve. Control incubations of fibrinogen in the absence of enzyme and enzyme in the absence of fibrinogen provided zero sialic acid values. Addition of standard quantities of N-acetylneuraminic acid to the incubation mixture resulted in 100% recovery. At the completion of incubation, the altered fibrinogen was precipitated with glycine (175.5 mg/ml solution) at 37°C. The precipitate was removed by centrifugation at 4000 *g* for 20 min, washed, and dissolved in a minimum volume of buffer-EACA. Total hexose assays of native fibrinogen and neuraminidase-treated fibrinogen were performed by the phenol-sulfuric acid method (36).

Preparation of acylated fibrinogen. Acylated fibrinogen was prepared by the acetic anhydride method in buffer devoid of EACA (37). This was followed by extensive dialysis

[1] *Abbreviations used in this paper:* EACA, epsilon-amino caproic acid; PGS, purified proteinpolysaccharide basic structural unit; PPL3, proteinpolysaccharide basic structural unit; PRP, platelet-rich plasma; PPP, plated-poor plasma.

against buffer-EACA, followed by human Ringer-0.1 mM EDTA. Free-amino groups were assayed on samples of native and acylated fibrinogen by the ninhydrin method (38).

Preparation of esterified fibrinogen. Fibrinogen solutions were first dialyzed against distilled water, lyophilized, and esterified by both the methanol-HCl method (39) and the propylene oxide method (40). The products obtained were consistently soluble in distilled water, but insoluble in human Ringer-0.1 mM EDTA, isotonic saline, or saline solutions as low as 0.085 g per 100 ml.

Preparation of fibrinogen modified at the tryptophan residue. Similarly, attempts to modify the tryptophan residues by treatment with *o*-Nitrophenylsulphenyl chloride (41) or with formic acid-HCl (42) resulted in products which were insoluble in human Ringer-0.1 mM EDTA. The N-bromosuccinimide procedure (43) resulted in precipitation within the reaction mixture itself.

Assay of thrombin time and anti-thrombin activity. For both measurements, the thrombin solution was adjusted to a concentration which gave a control thrombin time, at 37°C, for the purified fibrinogen of 6–12 sec. The thrombin time was measured by preincubating 0.12 ml of the incubation solution (plasmin digested fibrinogen) with 0.28 ml of 0.2 M EACA in 55 mM citrate buffer, pH 7, and then adding 0.1 ml of thrombin solution. The anti-thrombin activity was measured by preincubating 0.2 ml of the incubation solution with 0.2 ml of 0.2 M EACA in 55 mM citrate buffer, pH 7, containing 5 mg/ml native fibrinogen and adding 0.1 ml thrombin solution.

Preparation of other protein solutions. Serum was prepared from ACD platelet-poor plasma by addition of bovine thrombin at a concentration of 6–8 U/ml for 16 to 18 hr at 37°C. Serum was expressed from the clot and found to be devoid of residual thrombin by a sensitive platelet agglutination method (44). Bovine albumin was prepared free of adsorbed fatty acids by extracting with isooctane (45). A crude human globulin fraction was prepared as described previously (44). These proteins and all other proteins tested were dialyzed against three changes of human Ringer-0.1 mM EDTA at 4°C, and centrifuged at 35,000 *g* before use (discarding any insoluble material).

Preparation of washed leukocytes and erythrocytes. Human leukocytes were prepared by the method of Baehner, Karnovsky, and Karnovsky (46) and suspended in human Ringer-0.1 mM EDTA. Human erythrocytes were washed and suspended in the same solution.

Preparation of proteinpolysaccharide complexes and side chains. Proteinpolysaccharides were prepared from cartilage in 3 M $MgCl_2$ by recently described dissociative extraction techniques (47). The extracted proteinpolysaccharides were separated into discrete molecular species with 2.5 M potassium acetate and 2.5 M $CaCl_2$ (47). These species were characterized hydrodynamically by ultracentrifugal methods (47) and electron microscopy (48). Proteinpolysaccharide basic structural unit (PPL3) was separated from aggregate forms (PPL5 α and β) by differential ultracentrifugation in high molarity salt solutions (47, 49). Highly purified proteinpolysaccharide basic structural unit (PGS) was prepared by density gradient centrifugation (47, 50). The mucopolysaccharide side chains: chondroitin-4-sulfate, chondroitin-6-sulfate, and keratan-sulfate were prepared from proteinpolysaccharides by proteolytic degradation of the protein core, after fractionation of the barium salts of chondroitin and keratan-sulfate with ethanol (51).

MATERIALS

Fresh human platelets were obtained, prepared, and washed in human Ringer solution (containing 2–0.1 mM EDTA) as described previously (2). Human Ringer solution contains: 127 mM NaCl, 4 mM KCl, 25 mM $NaHCO_3$, 2 mM Na_2HPO_4, 0.2 mM Na_2SO_4, and is gassed with 95% O_2–5% CO_2 to a pH of 7.1. Collagen from bovine tendon, hexokinase from yeast (practical grade, type 3), porcine thyroglobulin (type 2), epsilon-aminocaproic acid, glycine, sodium citrate, N-acetylneuraminic acid, *o*-Nitrophenylsulphenyl chloride, and N-bromosuccinimide were obtained from Sigma Chemical Co., St. Louis, Mo. Neuraminidase from *Clostridium perfringens* (1.1 units/mg), and muramidase, were obtained from Worthington Biochemical Corp., Freehold, N. J. Analytical grade propylene oxide, acetic anhydride, and formic acid were obtained from Fisher Scientific Company, Springfield, N. J. Plastic cover slips (22 mm × 22 mm) were obtained from Arthur H. Thomas Company, Philadelphia, Pa.; and 2,2,4-Trimethylpentane (isooctane) from Phillips Petroleum Company, Bartlesville, Okla. Human plasmin, 23.3 casein U/ml, was a gift from Dr. Alan Johnson of The American National Red Cross Laboratory, New York. Topical bovine was obtained from Parke Davis & Co., South Hackensack, N. J. Bovine albumin (fraction V) was obtained from Armour Pharmaceutical Co., Kankakee, Ill.; salt free hemoglobin, from Mann Research Laboratories, Inc., New York; cytochrome-C from Boehringer and Soehne, New York; and U.S.P. regular insulin (80 U/ml) from Squibb Pharmaceuticals, New York. EDTA Vacutainer test tubes were obtained from Becton-Dickinson & Co., Rutherford, N. J. ACD-A (1.5 part to 10 parts whole blood) was obtained from Fenwal Laboratories, Inc., Morton Grove, Ill.

RESULTS

Fig. 1 demonstrates the typical appearance of the collagen-coated plastic surface employed in these studies. Panel A reveals the appearance after incubation in (0.5 volume % or approximately 390,000/mm^3) washed platelets suspended in human Ringer solution. It consists of a dense meshwork of thin collagen fibers embedded in a finely granular film of soluble collagen. No areas of the plastic coverslip are left uncovered by collagen. A few platelets are adherent to the collagen surface despite rinsing three times in human Ringer solution.

Panel B reveals the appearance of such a surface after incubation in (0.5 volume %) washed platelets suspended in plasma and rinsed three times in platelet-poor plasma. An increased number of platelets adhere to the collagen surface. These are not completely immobile. They are located in a plane focus slightly above that of the collagen fibers, and can be made to move as many as ten platelet diameters from their positions by gently blowing across the microscope slide. However, they appear to be anchored in that they will often return to their approximate original location.

Anticoagulant effect. Blood was obtained by venipuncture from three volunteers. One sample from each was anticoagulated with ACD and a second with EDTA.

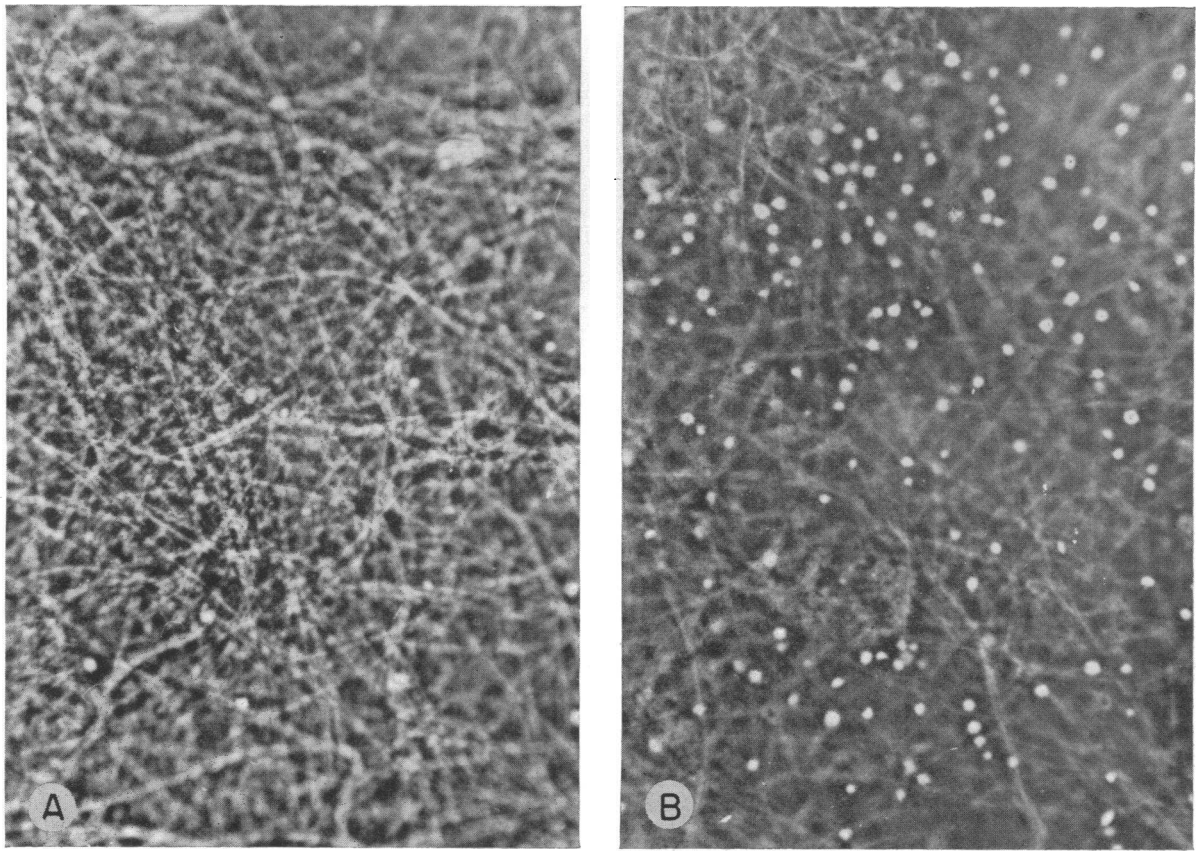

FIGURE 1 Adherence of human platelets to a collagen-coated plastic surface. (*A*) 0.5% volume suspension of washed platelets in human Ringer solution. (*B*) 0.5% volume suspension of washed platelets in platelet-poor plasma.

Platelet-rich plasma (PRP) was obtained by a single gentle centrifugation (320 *g* for 30 min) and the platelet adhesion assay performed in these solutions. In some experiments, an additional 0.5 volume % of washed platelets was added to the PRP in order to increase the platelet concentration. No difference could be detected in blood anticoagulated with ACD compared to the same blood anticoagulated with EDTA, regardless of platelet concentration.

Effect of suspending media on platelet adhesion. Table I lists the effect of various suspending media on the number of platelets which remain adherent to the collagen surface after incubation with a 0.5 % platelet suspension. Platelet-poor plasma, serum, albumin, and fibrinogen were considerably more effective than human Ringer solution in supporting platelet adhesion to the collagen surface. Few platelets adhere when they are suspended in the indicated concentrations of human globulin, hexokinase, hemoglobin, cytochrome-C, insulin, thyroglobulin, or muramidase. No correlation could be made with the physical properties (52) of the protein

TABLE I
Effect of Various Suspending Media on Platelet Adhesion

Solution		Platelets per grid
Human Ringer	(53)	6 ±0.7*
Platelet-poor plasma	(36)	20 ±1.8
Serum	(8)	14 ±2.7
Albumin, 45–50 mg/ml	(9)	14 ±1.6
Albumin, 10 mg/ml	(1)	6
Albumin (extracted), 50 mg/ml	(1)	18
Fibrinogen, 2.7–5.0 mg/ml	(42)	16 ±0.9
Globulin, 20–23 mg/ml	(6)	5 ±1.1
Hexokinase, 10 mg/ml	(2)	8
Hemoglobin, 50 mg/ml	(1)	6
Cytochrome-c, 2.5 mg/ml	(1)	5
Insulin, 1.4 mg/ml	(1)	4
Thyroglobulin, 10 mg/ml	(1)	3
Muramidase, 10 mg/ml	(1)	3

Number of experiments are given in parentheses.
* SEM.

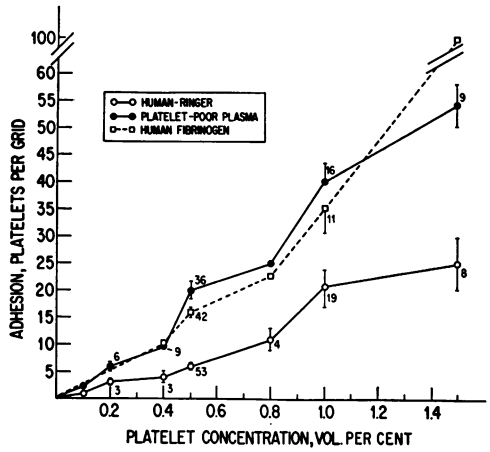

FIGURE 2 Effect of platelet concentration (0.1–1.5 volume %) on the adhesion of platelets to a collagen-coated surface after suspension in human Ringer, platelet-poor plasma anticoagulated with ACD or purified fibrinogen (2–4 mg/ml). Number of experiments and SEM are given.

solutions employed (molecular weight, isoelectric point, diffusion constant, frictional ratio) and their propensity to support adhesion. Further studies were performed to elucidate the nature of the adhesion enhanced by platelet-poor plasma and fibrinogen.

Effect of platelet concentration. Platelets were suspended in concentrations ranging from 0.1 to 2.0% by volume in human Ringer solution, platelet-poor plasma collected in ACD, and fibrinogen (2–4 mg/ml). Platelet adhesion was proportional to platelet concentration with all three suspending media, Fig. 2. A limit to the number of platelets which adhere was imposed only by the tendency of platelets at high concentration (above 2.0%) to aggregate in the presence of collagen surface.

TABLE II
*Adhesion of Platelets after Incubation at 37°C**

Experiment	Time of incubation	Platelet concentration	Average adhesion: Human Ringer solution	Average adhesion: Platelet-poor plasma	Average adhesion: Fibrinogen, 5 mg/ml
	min	*vol. %*	*platelets per grid*		
I	0	0.5	6	20	25
	0	1.0	20	41	36
II	30	0.5	6	20	31
	30	1.0	14	32	47
III	60	0.5	13	35	N.T.‡
	60	1.0	21	42	N.T.‡

* A 5 volume % platelet suspension was incubated for 0, 30, and 60 min at 37°C and then resuspended to concentrations of 0.5 and 1.0% before testing for platelet adhesion.
‡ Not tested.

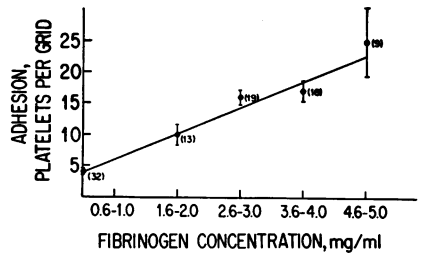

FIGURE 3 Effect of fibrinogen concentration (0–5 mg/ml) on platelet adhesion to a collagen-coated surface. Number of experiments are given in parentheses. SEM is given.

Both platelet-poor plasma and fibrinogen supported platelet adhesion to a greater extent than human Ringer solution.

Effect of temperature. The adhesion of a 0.5% suspension of platelets was tested in a fibrinogen solution (3.9 mg/ml) at 37°C. The same test vials were then cooled and maintained at 0–1°C in an ice-water bath, and the adhesion test repeated. No difference in adhesion was detected at the two temperatures, (21 vs. 19 platelets per grid at 0 and 37°C, respectively).

Effect of prior incubation of platelets (glycogen depletion). Platelets at a concentration of 5 volume % were incubated at 37°C in human Ringer solution for 0, 30, and 60 min[2] and then resuspended to concentrations of 0.5 and 10% in human Ringer solution, PPP, and fibrinogen (5 mg/ml). No apparent impairment of adhesion was detected, Table II.

Adhesion of leukocytes and erythrocytes. Washed leukocytes (95% granulocytes at a packed cell volume of 0.9%) and erythrocytes (at a packed cell volume of 0.5–6%) were suspended to various concentrations in human Ringer solution and fibrinogen (3–4 mg/ml), and the adhesion assay performed in a manner identical to that described for platelets. No significant leukocyte or erythrocyte adhesion to collagen was noted.

Adhesion of Platelets Suspended in Fibrinogen Solutions

Effect of fibrinogen concentration. Platelets at a concentration of 0.5% were suspended in fibrinogen solutions ranging in concentration from 0 to 6.5 mg/ml. Fig. 3 depicts the linear increase in platelet adhesion with increasing fibrinogen concentration. As with increasing platelet concentration, a limit to the number of platelets which adhere was imposed only at high fibrinogen concentration (> 6.5 mg/ml), by the tendency of 0.5% platelet suspensions to aggregate.

Fibrinogen degradation products. Solutions of fibrinogen-degradation products were classified on the basis of

[2] This incubation procedure decreases glycogen stores to less than 50% of freshly prepared platelets (2).

TABLE III

Adhesion of Platelets in Suspensions of Fibrinogen Degradation Products*

	Sequential digests					
	A‡	B	C	D	E	F
Adhesion, *per cent native fibrinogen*	100	82	69	58	38	31
Thrombin time, *sec above control*	0	6	13	19	20	>46
Ability to clot, %	95	84	74	56	29	0

* 0.5% platelet suspension.

‡ Undigested native fibrinogen, 4 mg/ml.

thrombin times, and clottability. The variation of both these parameters during the course of a typical plasmin digestion experiment is shown in Table III. The adhesion of platelets suspended in solutions of fibrinogen-degradation products was compared in each experiment to the adhesion of platelets suspended in a solution of fibrinogen at the same protein concentration and containing identical concentrations of EACA and plasmin. Each class of digest supported platelet adhesion to a significantly less extent than did fibrinogen alone,[3] Table III.

To determine the point at which the ability of fibrinogen digests to support adhesion diminished, the following experiment was designed: The clottable fibrinogen remaining in the digests was examined on SDS-polyacrylamide gel electrophoresis after reduction with mercaptoethanol. This procedure (34) is capable of distinguishing the fibrin chains α ($\alpha 1$ and $\alpha 2$), β and γ, Fig. 4. Intact fibrin is depicted in panel A. Panels B, C, D, and E refer to plasmin-digestion products with thrombin times (above control) of 6, 12, 19, and 20 sec. It is apparent that failure to support adhesion is associated with the disappearance of the α-chains and the appearance of the new β'-chain.

Neuraminidase-treated fibrinogen. Treatment of fibrinogen with neuraminidase released sialic acid accounting for 0.74 ±0.02% (SEM) by weight of fibrinogen (six experiments). No decrease in either clottability or total hexose content of fibrinogen occurred after treatment with neuraminidase. Repeated paired assays (20 experiments) comparing neuraminidase-treated fibrinogen with native fibrinogen failed to show any significant difference in their ability to support platelet adhesion, (13 vs. 16, respectively, SEM < 10%).

[3] Later digestion products (thrombin times > 100 sec and anti-thrombin times of > 80 to < 20 sec) often demonstrated a partial restoration of the ability to support adhesion. These results were inconsistent however, and this interesting observation not further pursued.

Acylated fibrinogen. Acylation of three batches of fibrinogen reduced the number of free-amino groups (leucine equivalents) available to the ninhydrin reagent by 20%. No change in clottability of the fibrinogen occurred after acylation. Paired assays (seven experiments) comparing acylated with native fibrinogen showed no significant difference in the adhesion of platelets suspended in the acylated products (19 vs. 16, respectively, SEM < 10%).

Effect of proteinpolysaccharides on platelet adhesion to collagen

Proteinpolysaccharides were as effective, or more effective, than fibrinogen in enhancing the adhesion of platelets to collagen.

Initially extracted, unfractionated proteinpolysaccharide from bovine nasal cartilage, containing a mixture of proteinpolysaccharide basic structural units and aggregate forms, was three to four times more effective than fibrinogen (by weight) in supporting platelet adhesion (Table IV). Its mucopolysaccharide moiety consists mainly of chondroitin-sulfate side chains. The purified proteinpolysaccharide basic structural unit PPL3 ($S°_{20,w} = 19.7S$) was as effective as fibrinogen (by weight). The higher molecular weight aggregate forms (PPL5 α, $S°_{20,w} = 40S$, PPL5 β, $S°_{20,w} > 100S$) were roughly twice as effective as fibrinogen (by weight).

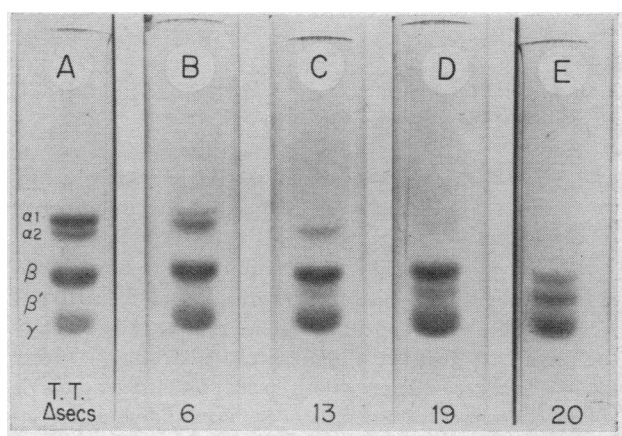

FIGURE 4 SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis of mercaptoethanol-treated clottable fibrinogen and fibrinogen degradation products. Fibrinogen or its degradation products were clotted with a final concentration of bovine thrombin of 7.5 U/ml in 0.015 M sodium phosphate, 0.075 M NaCl, 0.1 M EACA, pH 6.4 for 30 min at 20°C. The clottable protein was treated at 37°C with 1% mercaptoethanol–1% SDS in 0.01 M phosphate buffer,, 8 M urea, pH 7.0, for 2 hr. 15 μg of protein were applied to a 9% gel and electrophoresed at 7 ma per tube for 16 hr at room temperature. Gels were stained for 5 min with Coomassie brilliant blue and destained by washing in 7% acetic acid. For further details see Reference 65.

TABLE IV

Effect of Proteinpolysaccharides on Platelet Adhesion to Collagen

Product	Concentration	Adhesion* above Ringer control
	mg/ml	
Fibrinogen	2–4	15–19
Bovine nasal cartilage		
Unfractionated	3	45
Proteinpolysaccharide, PPL	2	50
	1.5	27
	1.0	17
	0.3	7
	0.03	3
Basic structural	2	15
Unit, PPL3	1	7
Basic structural	2	18
Unit, PGS	1	10
Aggregate	2	20
Forms (PPL5) $\alpha + \beta$	1	14
Bovine nucleus pulposus	2	8
Unfractionated proteinpolysaccharide	1	5
Bovine humeral articular cartilage	2	10
Unfractionated proteinpolysaccharide	1	5
Chondroitin 4-sulfate	2	3
Chondroitin 6-sulfate	2	2
Keratan-sulfate	2	3

* Number of platelets per grid.

The highly purified proteinpolysaccharide basic structural unit PGS ($S°_{20,w} = 22.1S$) was twice as effective as fibrinogen (by weight).

Proteinpolysaccharide preparations from bovine nucleus pulposus and bovine humeral articular cartilage, were capable of supporting platelet adhesion but not to the same extent as proteinpolysaccharides obtained from bovine nasal cartilage whose mucopolysaccharide side chains contain equal amounts of keratan and chondroitin-sulfate. Purified mucopolysaccharide side chains (chondroitin 4-sulfate, chondroitin 6-sulfate, and keratan-sulfate) were ineffective in supporting adhesion.

DISCUSSION

A procedure has been developed which has proved to be a reliable laboratory tool for the quantitation of platelet adhesion. This procedure is different from others in that: (*a*) it employs a purified collagen fiber system for the site of adhesion, (*b*) platelets adherent to collagen may be observed directly and quantitated; (*c*) a washed platelet system can be employed, so that various suspending media can be tested.

The data obtained from this procedure emphasize the importance of the suspending medium as a variable factor for platelet adhesion. Thus, washed platelets at physiologic platelet concentrations lose their ability to adhere to collagen. This ability is restored by the addition of platelet-poor plasma, albumin at 45–50 mg/ml, fibrinogen at 2–4 mg/ml or proteinpolysaccharide at 1–2 mg/ml. This is clearly not a nonspecific protein effect, since seven other proteins at comparable or higher concentrations, were incapable of supporting platelet adhesion. Platelet adhesion is very much dependent upon platelet concentration as well as fibrinogen concentration, both of which may vary significantly in various clinical disorders.

Support of platelet adhesion by fibrinogen is independent of the sialic acid moiety of fibrinogen as well as 20% of the free amino groups. It is of interest that the sialic acid moiety of fibrinogen has no effect on the conversion of fibrinogen to fibrin monomer, nor the rate of fibrin monomer polymerization (53, 54).

Digestion of the fibrinogen molecule with plasmin results in inability to support platelet adhesion with very early degradation products. This is paralleled by an increase in thrombin time and a decrease in clottability (thrombin times 6 to > 46 sec above control; clottability 84–0%, Table III). This is associated with the disappearance of the α1-chain of fibrinogen and appearance of the modified β-chain of fibrinogen. Later digestion products are often paradoxically associated with a partial restoration of the ability to support adhesion. This finding is intriguing and remains unexplained.

The requirement of fibrinogen for platelet adhesion to glass has been reported by Zucker and Vroman (55) and by Packham, Evans, Glynn, and Mustard (56). Vroman and Adams (57, 58) have shown that fibrinogen is selectively adsorbed from plasma within 2 sec after exposure to wettable oxidized silicon crystals or anodyzed tantalum slides. Similarly, Baer and Dutton (59) have used a variety of tools (infrared spectroscopy, ellipsometry, and contact angle measurements) to demonstrate the early deposition of a film of fibrinogen on the surface of germanium prisms exposed to fresh venous or arterial blood. This deposition preceded the adhesion of platelets to the surface (60). The data presented in our study are consistent with the hypothesis that fibrinogen is a plasma factor required for platelet adhesion to both glass and purified collagen. In this regard, it is of interest that fibrinogen is either required for, or considerably enhances ADP-induced platelet aggregation (61–63).

The ability of albumin to support platelet adhesion is of interest despite the fact that fibrinogen is ten to twenty

times more effective, since the effective concentration of albumin is in the physiologic range. This does not appear to be the result of adsorbed free fatty acids (which are capable of aggregating platelets (61, 63)) since albumin pretreated with isooctane (45) gave similar results. Since albumin is such a nonspecific binder of various plasma substances, it is conceivable that some contaminant was responsible for its ability to support adhesion.

Platelet adhesion to collagen was as effective in platelet-rich plasma anticoagulated with EDTA as in ACD, although the former binds divalent cations much more effectively. These observations are consistent with the report of Spaet and Zucker (22) that platelets do not require divalent cation for adhesion to collagen. However, divalent cations are apparently required for the adhesion of platelets to glass (22).

The ability of platelets to adhere to collagen is not diminished at low temperature or by depletion of over 50% of glycogen stores, indicating that significant metabolic energy is not required.

Support of platelet adhesion by intact proteinpolysaccharide macromolecules obtained from three different connective tissues, is of considerable interest. The intact, native proteinpolysaccharide macromolecule consists of a protein core 3400 Å long, with side chains of chondroitin-sulfate and/or keratan-sulfate which are approximately 500 Å long. Each side chain carries closely spaced negatively charged groups whose repelling forces maintain the side chain in an extended conformation. The native proteinpolysaccharide molecule is diffuse and occupies an enormous domain of solution (47, 48). Its effect on the adhesion of platelets to collagen may well be related to its physical characteristics in solution, rather than the chemical properties of its chondroitin or keratan-sulfate side chains. Since the molecular weight of the proteinpolysaccharde basic structural unit is ten times that of fibrinogen (47) the preparations are ten to thirty times more effective than fibrinogen on a molar basis, in supporting platelet adhesion.

Finally, it should be recognized that the 16–20 platelets adhering per grid (0.021/mm^2) probably represent a fraction of the total platelet population to which it has been exposed. It is conceivable that one is dealing with a select platelet population, possibly young platelets. In this regard, Hirsch, Glynn, and Mustard (66) have reported greater adhesiveness of young rabbit platelets to collagen. Similarly, Shulman, Watkins, Itsocoitz, and Students (67) and Karpatkin (68) have reported greater functional capacity for young platelets, compared to older platelets. In addition, we might be dealing with a refractory group of platelets, as noted by Rozenberg and Holmsen (69), with ADP (adenosinediphosphate) aggregation.

ACKNOWLEDGMENTS

Doctors L. Rosenberg (I-657), and S. Karpatkin (I-459), are Career Investigators of The Health Research Council of the City of New York.

Supported by a grant-in-aid from The New York Heart Association, and by United States Public Health Service Grant AM 0028.

We are indebted to The New York Blood Center for their cooperation in supplying fresh platelet-rich plasma.

REFERENCES

1. Haslam, R. J. 1968. Biochemical aspects of platelet function. Plenary Session Papers. *Proc. 12th Cong., Int. Soc. Hematol.* New York. 198.
2. Karpatkin, S., and R. M. Langer. 1968. Biochemical energetics of simulated platelet-plug formation. Effect of thrombin, adenosine diphosphate, and epinephrine on intra- and extra-cellular adenine nucleotide kinetics. *J. Clin. Invest.* **47**: 2158.
3. Murer, E. H. 1969. Clot reaction and energy metabolism in platelets. Effect and mechanism of inhibitors. *Biochim. Biophys. Acta.* **172**: 266.
4. Loder, P. B., J. Hirsch, and G. C. De Gruchy. 1968. The effect of collagen on platelet glycolysis and nucleotide metabolism. *Brit. J. Hematol.* **14**: 563.
5. Salzman, E. W., and L. Levine. 1971. Cyclic 3',5'-AMP in human blood platelets: II. Effect of N^6-2^1-0-dibutyrl cyclic 3^1,5^1-AMP on platelet function. *J. Clin. Invest.* **50**: 131.
6. Aledort, L. M., S. B. Troup, and R. I. Weed. 1968. Inhibition of sulfhydryl dependent platelet functions by penetrating and nonpenetrating analogues of parachloromercuribenzene. *Blood.* **31**: 471.
7. Lewis, N., and P. W. Majerus. 1969. Lipid metabolism in human platelets. II. De Novo phospholipid synthesis and the effect of thrombin on the pattern of synthesis. *J. Clin. Invest.* **48**: 2114.
8. Hugues, J. 1960. Accolement des plaquettes au collagéne. *C. R. Soc. Biol.* **154**: 866.
9. Bounameaux, Y. 1961. L'accolement des plaquettes aux fibres sousendothéliales. *Thromb. Diath. Haemorrh.* **6**: 504.
10. Hovig, T. 1963. Aggregation of rabbit blood platelets produced in vitro by saline extract of tendons. *Thromb. Diath. Haemorrh.* **9**: 248.
11. Baumgartner, H. R., J. P. Tranzer, and A. Studer. 1967. An electron microscopic study of platelet thrombus formation in the rabbit with particular regard to 5-hydroxytryptamine release. *Thromb. Diath. Haemorrh.* **18**: 592.
12. Tranzer, J. P., and H. R. Baumgartner. 1967. Filling gaps in the vascular endothelium with blood platelets. *Nature (London).* **216**: 1126.
13. Stemerman, M. B., H. R. Baumgartner, and T. H. Spaet. 1971. The subendothelial microfibril and platelet adhesion. *Lab. Invest.* **24**: 179.
14. Dische, Z. 1964. The glycans of the mammalian lens capsule—a model of basement membrane. *In* Conference on Small Blood Vessel Involvement in Diabetes Mellitus. M. D. Siperstein, A. R. Colwell, Sr., and K. Meyer, editors. American Instiute of Biological Sciences, Washington, D. C. 201.
15. Kefalides, N. A. 1968. Isolation and characterization of the collagen from glomerular basement membranes. *Biochemistry.* **7**: 3103.

16. Kefalides, N. A., and B. Denduchis. 1969. Structural components of epithelial and endothelial basement membranes. *Biochemistry.* **8:** 4613.
17. Zucker, M. B., and J. Borelli. 1962. Platelet clumping produced by connective tissue suspensions and by collagen. *Proc. Soc. Exp. Biol. Med.* **109:** 779.
18. Wilner, C. D., H. L. Nossel, and E. C. Le Roy. 1968. Aggregation of platelets by collagen. *J. Clin. Invest.* **47:** 2616.
19. Caen, J. P., and T. Legrand. 1968. L'analyse des fonctions plaquettaires. Adhésion et aggrégation des plaquettes au collagéne purifié. *Rev. Fr. Etud. Clin. Biol.* **13:** 1028.
20. Bankowski, E., S. Niewiarowski, and W. Rocowski. 1969. Decrease of platelets aggregating activity of soluble human collagen fractions during ageing. *Thromb. Diath. Haemorrh.* **21:** 441.
21. Hovig, T., L. Jorgensen, M. A. Packham, and J. F. Mustard. 1968. Platelet adherence to fibrin and collagen. *J. Lab. Clin. Med.* **71:** 29.
22. Spaet, T. H., and M. B. Zucker. 1964. Mechanism of platelet plug formation and role of adenosine diphosphate. *Amer. J. Physiol.* **206:** 1267.
23. Spaet, T. H., and I. Lejnieks. 1969. A technique for the estimation of platelet-collagen adhesion. *Proc. Soc. Exp. Biol. Med.* **132:** 1038.
24. Wright, H. P. 1942. Changes in the adhesiveness of blood platelets following parturition and surgical operations. *J. Pathol. Bacteriol.* **54:** 461.
25. Hellem, A. J. 1960. The adhesiveness of human blood platelets in vitro. *Scand. J. Clin. Lab. Invest.* **12:** (Suppl. 51).
26. O'Brien, J. R. 1961. The adhesiveness of native platelets and its prevention. *J. Clin. Pathol.* **14:** 140.
27. Salzman, E. W. 1963. Measurement of platelet adhesiveness. A simple in vitro technique demonstrating an abnormality in von Willebrand's Disease. *J. Lab. Clin. Med.* **62:** 724.
28. Sjogren, A., L. E. Bottiger, and G. Biorck. 1969. Platelet adhesion: a comparison of four methods. *Acta Med. Scand.* **185:** 127.
29. Aggeler, P. M. 1967. Report of the Subcommittee on Platelet-Glass Adhesion. *In* Platelets: Their Role in Hemostasis and Thrombosis. K. M. Brinkhous, I. S. Wright, J. P. Soulier, H. R. Roberts, and S. Hinnom, editors. F. K. Schattauer-Verlag, Stuttgart. 279.
30. Hartman, R. C. 1967. Platelet adhesiveness: the retention of platelets on glass surfaces. *In* Platelets: Their Role in Hemostasis and Thrombosis. K. M. Brinkhous, I. S. Wright, J. P. Soulier, H. R. Roberts, and S. Hinnom, editors. F. K. Schatauer-Verlag, Stuttgart. 281.
31. Shulman, N. R. 1967. Clinical experience with platelet adhesiveness test in von Willebrand's Disease. *In* Platelets: Their Role in Hemostasis and Thrombosis. K. M. Brinkhous, I. S. Wright, J. P. Soulier, H. R. Roberts, and S. Hinnom, editors. F. K. Schattauer-Verlag, Stuttgart. 309.
32. Kazal, L. A., S. Amsel, O. P. Miller, and L. M. Tocantins. 1963. The preparation and some properties of fibrinogen precipitated from human plasma by glycine. *Proc. Soc. Exp. Biol. Med.* **113:** 189.
33. Mills, D. A., R. Coyne, B. Pollara, and R. W. Von Korff. 1964. The action of plasmin on fibrinogen and fibrin. I. Changes in the N-terminal residues. *Biochim. Biophys. Acta.* **86:** 527.
34. Mills, D. A., and S. Karpatkin. 1970. Heterogeneity of human fibrinogen: possible relation to proteolysis by thrombin and plasmin as studied by SDS-polyacrylamide gel electrophoresis. *Biochem. Biophys. Res. Commun.* **40:** 206.
35. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234:** 1971.
36. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28:** 350.
37. Fraenkel-Conrat, H., R. C. Bean, and H. Lineweaver. 1949. Essential groups for the interaction of ovomucoid (egg-white trypsin inhibitor) and trypsin, and for tryptic activity. *J. Biol. Chem.* **177:** 385.
38. Moore, S., and W. H. Stein. 1948. Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.* **176:** 376.
39. Fraenkel-Conrat, H., H. S. Olcott. 1945. Esterification of proteins with alcohols of low molecular weight. *J. Biol. Chem.* **161:** 259.
40. Fraenkel-Conrat, H. 1944. The action of 1,2-epoxides on proteins. *J. Biol. Chem.* **154:** 227.
41. Scoffone, E., A. Fontana, and R. Rocchi. 1968. Sulphenyl halides as modifying reagents for polypeptides and proteins. I. Modification of tryptophan residues. *Biochemistry.* **7:** 971.
42. Proviero, A., M. A. Coletti-Previero, and J. Cavadore. 1967. A reversible chemical modification of the tryptophan residue. *Biochim. Biophys. Acta.* **147:** 453.
43. Spande, T. F., and B. Witkop. 1967. Reactivity toward n-bromosuccinimide as a criterion for buried and exposed tryptophan residues in proteins. *Methods Enzymol.* **11:** 528.
44. Karpatkin, S., and G. W. Siskind. 1969. In vitro detection of platelet antibody in patients with idiopathic thrombocytopenic purpura and systemic lupus erythematosus. *Blood.* **33:** 795.
45. Goodman, D. S. 1957. Preparation of human serum albumin free of long-chain fatty acids. *Science (Washington).* **125:** 1296.
46. Baehner, R. L., M. J. Karnovsky, and M. L. Karnovsky. 1969. Degranulation of leukocytes in chronic granulomatous disease. *J. Clin. Invest.* **48:** 187.
47. Rosenberg, L., S. Pal, R. Beale, and M. Schubert. 1970. A comparison of proteinpolysaccharides of bovine nasal cartilage isolated and fractionated by different methods. *J. Biol. Chem.* **245:** 4112.
48. Rosenberg, L., W. Hellman, and A. K. Kleinschmidt. 1970. Macromolecular models of proteinpolysaccharides from bovine nasal cartilage based on electron microscopic studies. *J. Biol. Chem.* **245:** 4123.
49. Pal, S., P. T. Doganes, and M. Schubert. 1966. The separation of new forms of the proteinpolysaccharides of bovine nasal cartilage. *J. Biol. Chem.* **241:** 4261.
50. Hascall, V. C., and S. W. Sajdera. 1969. Proteinpolysaccharide complex from bovine nasal cartilage. The function of glycoprotein in the formation of aggregates. *J. Biol. Chem.* **244:** 2384.
51. Rosenberg, L., M. Schubert, and J. Sandson. 1967. The proteinpolysaccharides of bovine nucleus pulposus. *J. Biol. Chem.* **242:** 4691.
52. West, E. S., W. R. Todd, H. S. Mason, J. T. Van Bruggen. 1966. Textbook of Biochemistry. The MacMillan Company, New York. 4th edition. 326.

53. Bray, B. A., and B. Alexander. 1969. Clotting of factor I (fibrinogen) treated with sialidase. *Fed. Proc.* **28**: 321. (Abstr. 356)
54. Mester, L., E. Moczar, G. Vass, and L. Szabados. 1965. Structure et role des fractions glucidiques du fibrinogéne. *Pathol. Biol.* **13**: 540.
55. Zucker, M. B., and L. Vroman. 1969. Platelet adhesion induced by fibrinogen adsorbed onto glass. *Proc. Soc. Exp. Biol. Med.* **131**: 318.
56. Packham, M. A., G. Evans, M. F. Glynn, and J. F. Mustard. 1969. The effect of plasma proteins on the interaction of platelets with glass surfaces. *J. Lab. Clin. Med.* **73**: 686.
57. Vroman, L., and A. L. Adams. 1967. Possible involvement of fibrinogen and proteolysis in surface activation. *Thromb. Diath. Haemorrh.* **18**: 510.
58. Vroman, L., and A. L. Adams. 1969. Identification of rapid changes at plasma–solid interfaces. *J. Biomed. Mater. Res.* **3**: 43.
59. Baer, R. E., and R. C. Dutton. 1969. Initial events in interactions of blood with a foreign surface. *J. Biomed. Mater. Res.* **3**: 191.
60. Dutton, R. C., T. J. Webber, S. A. Johnson, and R. E. Baier. 1969. Microstructure of initial thrombus formation on foreign materials. *J. Biomed. Mater. Res.* **3**: 13.
61. Haslam, R. J. 1964. Role of adenosine diphosphate in the aggregation of human blood platelets by thrombin and by fatty acids. *Nature (London)*. **202**: 765.
62. Brinkhous, K. M., M. S. Read, and N. F. Rodman. 1969. Need of fibrinogen for thrombin-induced aggregation of porcine platelets. *J. Lab. Clin. Med.* **73**: 1000.
63. Deykin, D., C. R. Pritzker, and E. M. Scolnick. 1965. Plasma cofactors in adenosine diphosphate induced aggregation of human platelets. *Nature (London)*. **208**: 296.
64. Mahadevan, V., H. Singh, and W. O. Lundberg. 1966. Effects of saturated and unsaturated fatty acids on blood platelet aggregation in vitro. *Proc. Soc. Exp. Biol. Med.* **121**: 82.
65. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406.
66. Hirsch, J., M. F. Glynn, and J. F. Mustard. 1968. The effect of platelet age on platelet adherence to collagen. *J. Clin. Invest.* **47**: 466.
67. Shulman, N. R., S. P. Watkins, Jr., S. B. Itsocoitz, and A. B. Students. 1968. Evidence that the spleen retains the youngest and hemostatically most effective platelets. *Trans. Ass. Amer. Physicians.* **81**: 302.
68. Karpatkin, S. 1969. Heterogeneity of human platelets. II. Functional evidence suggestive of young and old platelets. *J. Clin. Invest.* **48**: 1083.
69. Rosenberg, M. C., and H. Holmsen. 1968. Adenine nucleotide metabolism of blood platelets. IV. Platelet aggregation response to exogenous ATP and ADP. *Biochim Biophys. Acta.* **157**: 280.