Inhibition of Collagen-Induced Platelet Aggregation by Normal Plasma

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ABSTRACT Normal plasma has been found to inhibit the platelet aggregation-inducing effect of collagen in a time consuming reaction independent of temperature. Collagen treated with serum and washed has reduced reactivity which can be restored to normal by treatment with 1.5 M sodium chloride. On the basis of this result, it is suggested that inhibition results from adsorption to collagen of a plasma component. The inhibitory plasma component is destroyed at 56°C, is unstable below pH 7, and migrates with the alpha globulins on starch block electrophoresis at pH 8.6. On the basis of ultrafiltration and sucrose density gradient ultracentrifugation studies, a molecular weight in the range of 330,000 is suggested and there may be an additional component of considerably greater size. Partial purification can be achieved by ion exchange chromatography. The purified fraction was completely inactivated by incubation with trypsin. Partially purified fractions inhibit cationic platelet aggregators such as collagen, polylysine, and hexadimethrine but do not affect anionic aggregators such as succinylated collagen and sodium stearate. Normal plasma and serum inhibit succinylated collagen and stearate. Stearate is inhibited by crystalline albumin and Cohn fraction IV-4. It is suggested that plasma proteins may regulate platelet adhesion to collagen and other vessel wall materials.

INTRODUCTION

There is much experimental evidence that platelet interaction with vessel wall components is an important part of the normal hemostatic mechanism. Of the vessel wall constituents, the evidence concerning collagen is the best established (1) but there are suggestions that basement membrane (2) and microfibrils [1] may also react with platelets. Purified collagen fibers cause platelet aggregation in vitro by a series of reactions which include initial adhesion of platelets to collagen and release of adenosine diphosphate by platelets (1, 3). Conditions quantitatively influencing the development of platelet aggregates include the intensity of the stimulus, and the reactivity of the platelets. In the present study, a previously unrecognized constituent of normal plasma is described which binds to collagen and inhibits platelet adhesion and aggregation. Previous studies presented evidence that the free amino groups of lysine are critical for the platelet aggregating effect of collagen (4). It was also shown that some materials causing platelet aggregation could be divided into two categories, one dependent for activity on positively charged groups and another on negatively charged groups (5). The purified plasma inhibitor described in this publication neutralizes the cationic aggregators but not the anionic group.

METHODS

Platelet-rich plasma was collected and prepared as previously described (4) and kept at room temperature until it was tested.[2] The blood was anticoagulated with 0.1 volume 4% trisodium citrate or 1% (w/v) heparin (Hynson, Westcott & Dunning, Inc., Baltimore, Md.). Platelet-poor plasma was prepared by centrifuging platelet-rich plasma until the count was less than 100 platelets per mm^3. Serum was prepared from platelet-poor plasma as previously described (18) except that the test tubes were not treated with silicone. Saline (0.15 M), buffered with barbital acetate at pH 7.40 was prepared as previously described (6). Other buffers were made as described by Gomori (7). Collagen was extracted from human cadaver skin by the Nishihara method as previously described (8). For platelet aggregation studies, the collagen was suspended in buffered saline using a Duall tissue grinder (Kontes Glass Co., Vineland, N. J.) (4). Succinylated collagen was prepared by treating Nishihara collagen with succinic anhydride by the method of Gustavson as described elsewhere (9). All the free amino groups on the collagen molecule were succinylated in the preparations tested (9). Reagent grade chemicals were used throughout

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[1] Baumgartner, H. R. 1970. Personal communication.

[2] Platelets were counted by the Rees-Ecker method (17).

and were obtained from the following suppliers: Cohn fractions I-VI and crystalline albumin (three times crystallized) both of human origin from Nutritional Biochemicals Corp., Cleveland, Ohio; the Cohn fractions were dissolved in saline buffer, dialyzed overnight against saline buffer and tested at concentrations of 1–10 mg/ml; L-polylysine (LY-105, mol wt 79,600) from Miles Labs, Inc, Elkhart, Ind.; sodium stearate from Price, Bromborough Pool, New Ferry, England; hexadimethrine bromide from Abbott Laboratories, North Chicago, Ill. Connective tissue was prepared as described by Zucker and Borrelli (10) and used in a concentration of 0.5 mg/ml.

Platelet aggregation was measured by a turbidimetric method (4). $\frac{1}{10}$ ml collagen or other platelet aggregator and 0.4 ml material to be tested for inhibitor activity were incubated together with consant stirring for varying time periods. In most experiments the preincubation was at room temperature but platelet aggregation was always recorded at 37°C. Quantitation of inhibitory activity was obtained by comparing the inhibitory effect of at least three dilutions of control and test materials. For stability and purification experiments, the starting plasma was arbitrarily designated as having an inhibitory activity of 1 unit per ml.

Platelet adhesion to collagen was tested by incubating 0.1 ml collagen (20 μg) with 0.4 ml saline or with 0.4 ml of a partially purified inhibitor preparation for 20 min with constant stirring. 1 ml platelet-rich plasma anticoagulated with disodium EDTA (3.9 mM, 2 mg/1.5 ml in the final mixture) was added and the mixture stirred at 37°C for 1 min. The samples were then examined by phase contrast microscopy (magnification × 3200, Zeiss photomicroscope II, Carl Zeiss Inc, New York) and adhesion or absence of adhesion to collagen was noted.

The effect of adsorbing serum to and eluting it from collagen was tested as follows: two 50 mg portions of the same batch of collagen were suspended in 7 ml pH 7.4 saline buffer. 200 ml saline was added to one portion termed the control and this batch was then treated in an identical way to the serum-treated collagen. 200 ml serum was added to the other collagen portion termed the serum-treated collagen. After stirring at room temperature for 20 min the collagen batches were centrifuged at 10,000 g for 15 min and the supernatant fluid was decanted. The collagen was resuspended in 10 ml distilled water and separated by centrifugation as described above. The washing with distilled water was repeated for a total of four times. The two collagen preparations were then lyophilized, suspended in saline, and platelet aggregating activity was measured in terms of the original batch of collagen. Each batch of collagen was then dialyzed against pH 7.4 1.5 M saline for 60 hr. The collagen was then separated by centrifugation and washed four times with 1.5 M saline. The two collagen batches were then dialyzed overnight against distilled water, lyophilized, suspended in 15 M saline, and tested for platelet aggregating activity in terms of the original batch of collagen.

The heat stability of the inhibitor was measured by heating 1 ml portions of serum in test tubes to different temperatures for 10 min. The sera were then transferred to an ice bath and inhibitory activity was measured in terms of the activity of a serum portion maintained in the ice bath throughout. The pH stability of the inhibitor was measured by dialyzing 2-ml portions of serum against various pH buffer solutions for 5 hr and then for 16 hr against 0.15 M saline buffered with barbital at pH 7.4. The inhibitory activity of the different serum samples was measured in terms of the activity of a serum portion which had been dialyzed against pH 7.4 buffer for 21 hr. 4-ml serum samples were dialyzed for 22 hr against large volumes of 0.025 M barbital buffer pH 7.4 with varying molarity sodium chloride (0–1 M). The samples were then centrifuged at 20,000 g for 15 min and the supernatant fractions decanted. Precipitates in samples containing 0.025 M sodium chloride and lower ionic strength were dissolved in 0.15 ml sodium chloride buffer. All samples were then dialyzed overnight against 0.15 M sodium chloride buffered with barbital at pH 7.4 and inhibitor activity was measured in terms of the activity of a sample initially dialyzed against buffered 0.15 M sodium chloride. Serum was separated into lipoprotein and nonlipoprotein fractions as follows: 10 ml serum was dialyzed against saline buffer for 4 hr at 4°C. 3.48 g KBr was added and the specific gravity adjusted to 1.021 by adding approximately 1.5 ml saline. The serum was centrifuged for 48 hr at 4°C at 40,000 g on a number 47 Beckman rotor (Beckman Instruments Inc, Palo Alto, Calif.) (19). The upper layer of lipoproteins (3 ml) was removed with a Pasteur pipet and upper and lower layers were dialyzed against buffered saline for 3 hr and tested for inhibitory activity.

Adsorption of serum was carried out for 5 min at 37°C. The materials tested for adsorption were Celite 512 (Johns-Manville Products Corp, New York) 20 mg per ml, aluminum hydroxide gel (Cutter Laboratories, Inc, Berkeley, Calif.) 0.1 ml per ml, $BaSO_4$ (J. T. Baker Chemical Co, Phillipsburg, N. J.) 100 mg per ml. Dialyses were at 4°C using VisKing dialysis tubing. Serum was ultrafiltered at room temperature with the use of apparatus from the Amicon Corp, Lexington, Mass.

Inhibitor fractions were ultracentrifuged at 78,000 g for 18 hr at 4°C on a Beckman model L-2 Spinco ultracentrifuge using an SW 39L rotor. 1 ml partially purified inhibitor fraction in saline, buffered at pH 8.6 or serum buffered at pH 8.6 was applied to 4 ml 10–40% sucrose gradient buffered with barbital at pH 8.6. After centrifuging three tubes with similar contents, the fluid in each tube was collected in five 1-ml fractions through a pinhole at the bottom of the cellulose acetate tube. The corresponding fractions from each of the three tubes were pooled, lyophilized, reconstituted with 1/3 volume distilled water, and dialyzed for 3 hr against buffered saline before being tested for inhibitory activity. In some of the ultracentrifuge runs, two of the tubes contained a partially purified inhibitor fraction or serum and the third tube contained a molecular weight marker. The following molecular weight markers were used, ovalbumin (45,000), aldolase (158,000), gamma globulin (160,000), thyroglobulin (670,000), all obtained from Mann Research Labs Inc, New York, fibrinogen (330,000) from Dr. R. E. Canfield and 28S ribosomes (1.6 million mol wt) from rabbit reticulocytes from Drs. M. Terada and P. A. Marks. Serum was electrophoresed at pH 8.6 on Pevikon (Pevikon C-870, Mercer Chemical Corporation, New York) as described by Müller-Eberhard and Osterland (11). 4.5 ml serum mixed with an equal volume of 0.1 M pH 8.6 barbital buffer was electrophoresed for 22 hr at 4°C. The block was sectioned and eluted with the starting buffer. The eluates were reduced in volume by air drying, dialyzed against pH 7.4 buffered saline and tested.

Ion exchange chromatography was carried out using DE52 (Whatman, from H. Reeve Angel & Co, Inc, Clifton, N. J.). 5 ml portions from the eluted fractions were pooled, lyophilized, reconstituted with 0.1 volume distilled water, dialyzed against 0.15 M pH 7.4 barbital buffered saline and tested for inhibitor activity. The contents of individual tubes from the active pool were then tested. The sensitivity of the inhibitor to trypsin was tested as follows: 1 ml inhibitor

partially purified by DEAE chromatography (specific activity 1.4 μ/mg, protein concentration 3.4 mg per ml) was incubated with 0.1 ml $CaCl_2$ 0.114 M and 4 mg trypsin (220 μ/mg, Worthington Biochemical Corp, Freehold, N. J.) at pH 8.1 for 2 hr at 25°C. 9 ml barbital-buffered saline pH 7.4 was added and the mixture ultrafiltered to a volume of 1 ml through an XM 100 Amicon membrane (retains molecules of 160,000 mol wt and greater). Two further 9-ml volumes were added to the retained fluid and filtered through the same membrane. The retained material was then assayed for inhibitory activity as compared with a control sample to which 0.1 ml saline was added in place of trypsin. Protein concentration was measured as described by Lowry, Rosebrough, Farr, and Randall (12).

RESULTS

Incubation of platelet-poor plasma with collagen resulted in progressive inactivation of the platelet-aggregating effect of collagen (Fig. 1a). Similar results were obtained whether the platelet-poor plasma was anticoagulated with heparin or citrate. Anticoagulation of the platelet-rich plasma with citrate or heparin also did not affect the results. The rate of inactivation of the collagen by plasma was independent of temperature between 4°C and 37°C. Using a constant incubation period the degree of inactivation of the collagen was related to the concentration of inhibitor fraction (Fig. 1b) or plasma

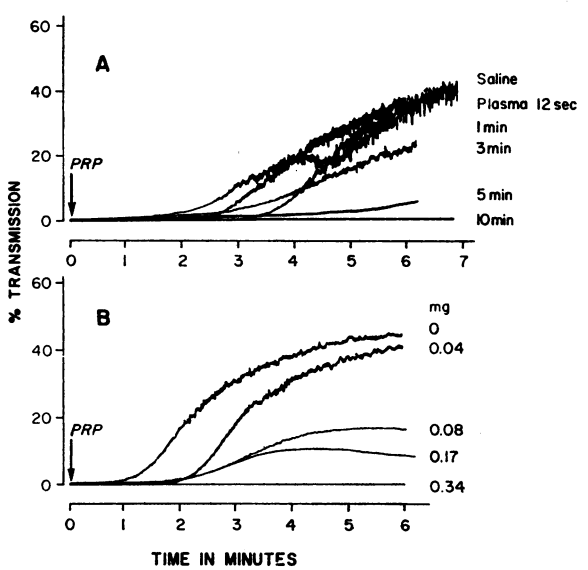

FIGURE 1 *The effect of incubation time and of different concentrations of inhibitor fraction on collagen-induced platelet aggregation.* (a). Normal plasma was incubated with 20 μg collagen for various time periods as indicated and 1 ml platelet-rich plasma was then added. (b). In place of normal plasma 0.4 ml inhibitor fraction was incubated with collagen for 5 min at 37°C before adding platelet-rich plasma. The inhibitor fraction used was prepared by DEAE chromatography as described in Fig. 4 and Table II. The specific activity of the fraction used in the experiment was 1.4 U/mg.

TABLE I

Activity of Collagen Treated with Serum, Eluted with Distilled Water, and then with 1.5 M Saline

	Activity
	%
Collagen incubated with 0.15 M saline, washed with distilled water, and lyophilized	100
Same collagen preparation dialyzed against 1.5 M saline, washed with 1.5 M saline, dialyzed against distilled water, and lyophilized	90
Collagen incubated with serum, washed with distilled water, and lyophilized	20
Serum treated collagen dialyzed against 1.5 M saline, washed with 1.5 M saline, dialyzed against distilled water, and lyophilized	120

incubated with the collagen. Although low inhibitor concentrations did delay the onset of the aggregation curve, higher concentrations affected the amplitude of the aggregation curve more than the delay period before the onset of aggregation. By testing the lowest concentration of plasma or fraction which completely inhibited the platelet aggregating activity of 20 μg collagen it was possible to quantitate the inhibitory activity of fractions. Serum was 80–100% as active as plasma. When collagen was incubated with serum and then washed with distilled water the collagen was one-fifth as active as control collagen. The original activity of the collagen was restored by treatment with 1.5 M saline (Table I).

Fractionation of plasma by precipitation with ammonium sulfate gave poor recovery of inhibitory activity. Inhibitory activity was not affected by dialysis against concentrated sodium chloride (to 1 M) but was lost after dialysis against hypotonic solutions (below 0.025 M sodium chloride) at pH 7.4.

The inhibitory activity was not adsorbed by celite or barium sulfate but was adsorbed by aluminum hydroxide gel. When tested for thermal stability most of the inhibitory activity was lost after 10 min at 56°C and complete inactivation occurred at 60°C. The inhibitor lost activity in 24 hr at 4°C but appeared to be stable at − 50°C and was not altered by freezing and thawing up to three times. Exposure to a pH range revealed marked loss of activity below pH 7 but stability at pH 7.4 to pH 10. Incubation of partially purified inhibitor with trypsin resulted in complete inactivation.

Equilibrium sedimentation of serum in KBr revealed inhibitory activity in the lower fraction and not in the upper indicating that the inhibitor does not behave as a lipoprotein. Cohn fractions I, II, III, IV-1, V, and VI from human plasma had no inhibitory effect in concentrations ranging from 1 to 10 mg per ml.

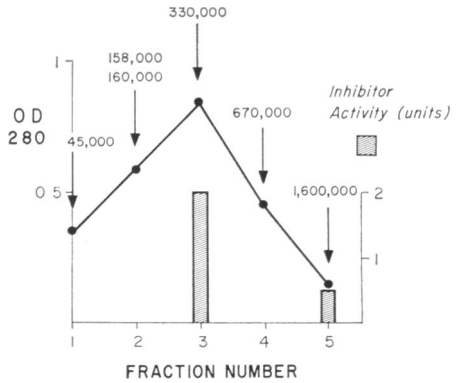

FIGURE 2 Sucrose density gradient centrifugation of partially purified inhibitor fractions. The fractions were prepared by ion exchange chromatography and the specific activity was 2.2 units per mg. The results are the mean derived from three separate ultracentrifugations. The molecular weight markers used were ovalbumin (45,000), aldolase (158,000) and gamma globulin (160,000), fibrinogen (330,000), thyroglobulin (670,000) and 28S ribosomes from rabbit reticulocytes (1.6 million). Inhibitory activity is shown by the bars. Fractions 1, 2, and 4 were devoid of inhibitory activity. ●——●, OD at 280.

The inhibitor was not dialyzable and ultrafiltration of serum indicated that the inhibitory activity was retained by the XM100 membrane in accordance with a molecular weight greater than 100,000. When a partially purified inhibitor fraction or serum was ultracentrifuged on a sucrose density gradient most of the inhibitory activity sedimented at approximately the same rate as fibrinogen but some inhibitory activity sedimented as a larger molecule (Fig. 2). On Pevikon block electrophoresis the inhibitor migrated with the alpha globulins (Fig. 3). Partial purification of the inhibitor could be achieved by ion exchange chromatography on diethylaminocellulose (Fig. 4, Table II). Recovery of activity was consistently greater after ion exchange chromatography than in the original plasma. The purified fraction was used to examine aspects of the inhibitory action. Mixture of the inhibitor with platelet-rich plasma suppressed platelet aggregation (Fig. 5). This result indicates that in the presence of a sufficiently high inhibitor concentration preincubation with collagen is not necessary to prevent platelet aggregation. Addition of the inhibitor to

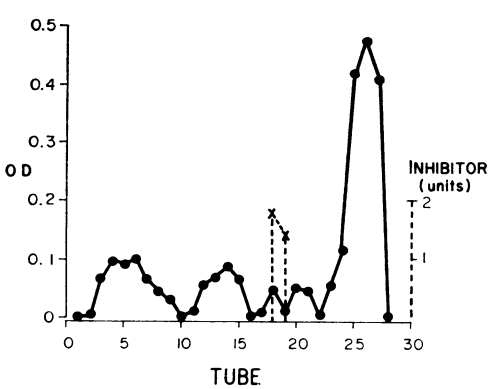

FIGURE 3 Electrophoretic behavior of inhibitor. The starch block was divided into 28 segments each of which was eluted with saline. The 280 OD of the eluates is shown by ●——● and inhibitory activity by ×---×.

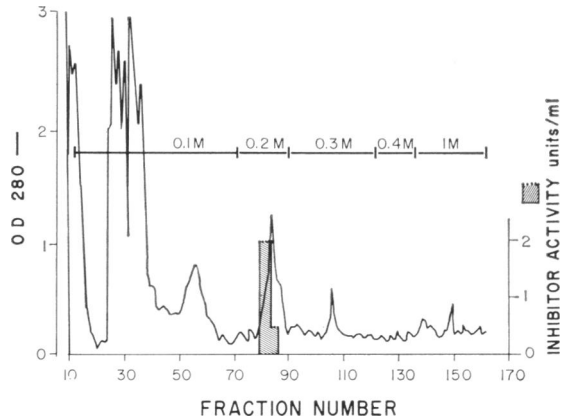

FIGURE 4 The elution of inhibitory activity from DE52 cellulose. Serum was dialyzed against 0.025 M barbital buffer pH 8.6 and applied to a DEAE column equilibrated with the same buffer. The protein was eluted with increasing molarity NaCl buffered with barbital at pH 8.6. The column size was 50 × 2·5 cm and 10 ml fractions were collected.

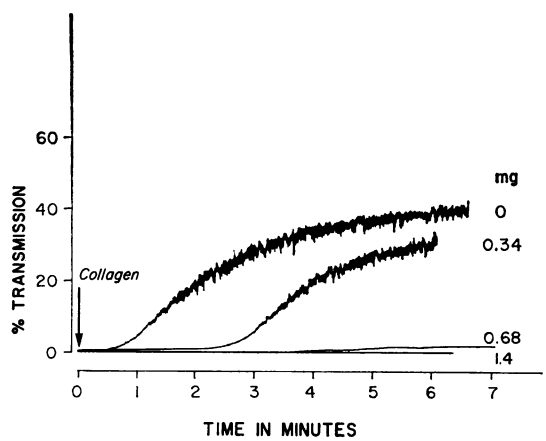

FIGURE 5 *The effect of partially purified plasma inhibitor on collagen-induced platelet aggregation.* The aggregation mixture of: 0.1 ml collagen (20 μg), 1.4 ml heparinized platelet-rich plasma mixture consisting of 1 ml platelet-rich plasma, and 0.4 ml of varying amounts of inhibitor fraction. The amount of inhibitor fraction added is shown next to the curves on the figure (the same fraction was used as in Fig. 1b).

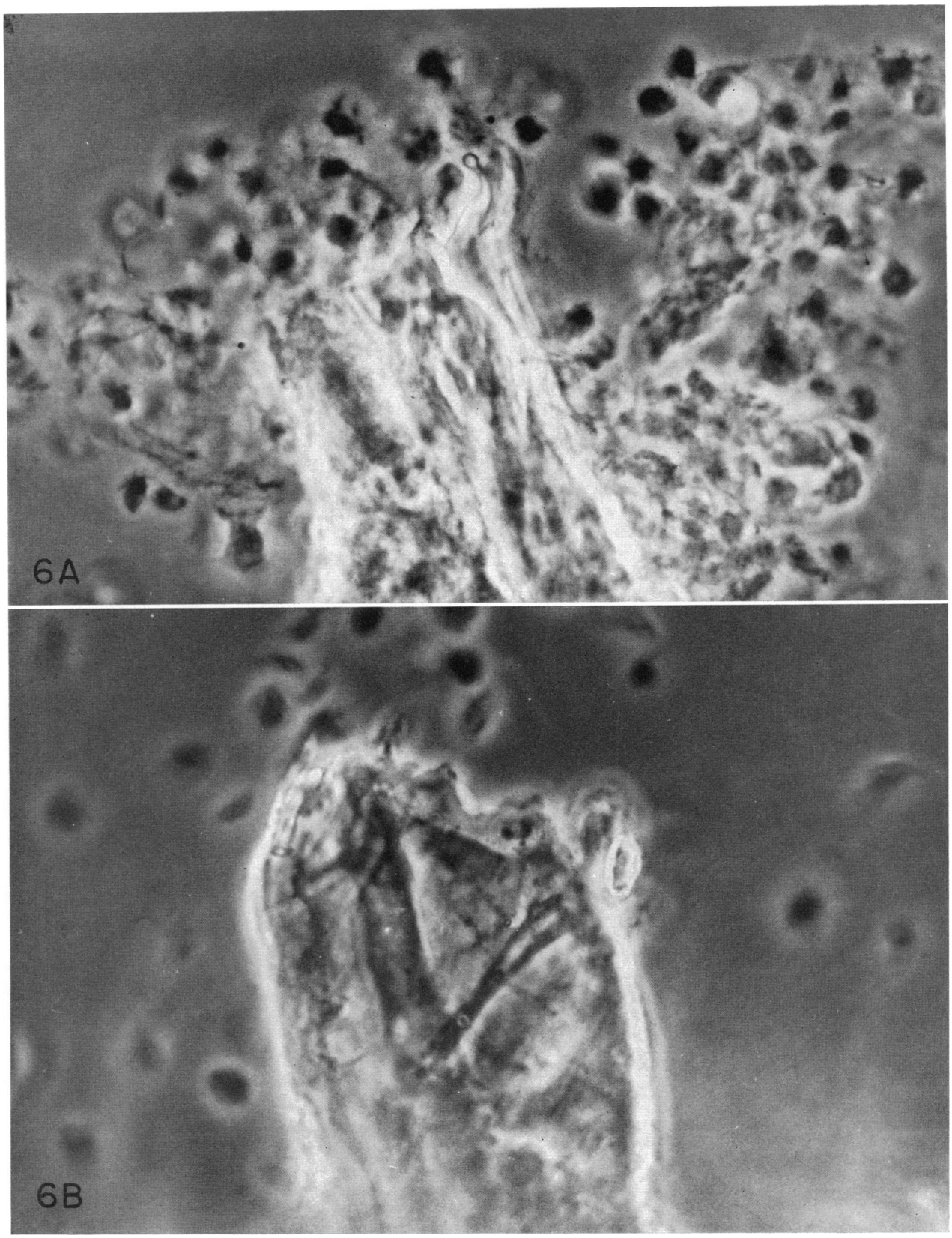

FIGURE 6 Photomicrographs of platelets added to connective tissue treated with saline (*a*) or plasma inhibitor (*b*).

TABLE II

Purification of Plasma Inhibitor by DEAE Chromatography

Material	Volume	Concentration of activity	Total activity	Protein concentration	Total protein	Specific activity	Yield	Purification
	ml	*μ/ml*	*μ*	*mg/ml*	*mg*	*μ/mg*	%	
Plasma	28	1	28	70	1980	0.014	100	1
Tubes 80–83	40	2	80	0.54	21.6	3.7	>100	264
Tubes 84–86	40	0.5	20	0.90	36	0.56	70	40

The Table gives the inhibitory activity, protein concentration, and volumes for the inhibitor recovered by DEAE chromatography described in Fig. 4.

TABLE III

Effect of Plasma and Plasma Fractions on Cationic and Anionic Platelet Aggregators

Cationic inducers of platelet aggregation	Marked inhibition	Slight inhibition	No inhibition
Collagen	Plasma inhibitor Whole plasma and serum		Albumin Cohn fractions I, II, III, IV-1, IV-4, V, VI
Polybrene	Plasma inhibitor	Albumin Whole serum	Cohn fractions I, II, IV-4
Polylysine	Plasma inhibitor	Whole serum Cohn fractions II, IV-4, V	Cohn fraction I
Anionic inducers of platelet aggregation			
Succinylated collagen		Whole plasma and serum	Plasma inhibitor Albumin Cohn fractions I, II, IV-4, V
Sodium stearate	Whole plasma and serum Albumin Cohn fraction IV-4		Plasma inhibitor

collagen effectively prevented platelet adhesion to collagen (Fig. 6).

The effect of a number of plasma proteins was tested on the platelet aggregating ability of materials dependent for activity on anionic or cationic groups. The plasma inhibitor neutralized the action of all three cationic materials and of crude connective tissue but was without effect on the anionic aggregators (Table III). Whole plasma and serum inhibited all the materials to a slight extent. Polylysine (50 μg) was inhibited to a slight extent by 6 mg Cohn fractions II, IV-4, and V (Table III). Neither crystalline albumin or the Cohn fractions tested affected the activity of succinylated collagen. Crystalline albumin neutralized sodium stearate in a 1 : 24 molar ratio (95 mg albumin neutralizes 1 mg stearate) and 2 μg Cohn fraction IV-4 neutralized 7 μg sodium stearate (Table III). The data on neutralization with albumin accords with the Goodman's (13) finding that albumin has 27 binding sites per molecule for stearate.

DISCUSSION

The result of the experiment shown in Fig. 1a indicates that platelet-free normal plasma neutralizes the platelet-aggregating effect of collagen in a time consuming reaction. The result of the experiment shown in Fig. 1b indicates that inhibition depends on the quantity of inhibitory material present. Since the rate of the inhibitory reaction was independent of temperature it is suggested that the reaction between inhibitor and collagen is physical rather than enzymatic. This suggestion is confirmed by the finding that serum-treated collagen remained less active after washing with distilled water but recovered full activity when washed with 1.5 M sodium chloride. On the basis of the results of that experiment it is further suggested that the inhibitor adsorbs to the collagen molecule probably by ionic bonds. Since the purified inhibitor neutralized the activity of all the cationic aggregators tested but did not affect the activity of the anionic aggregators it seems likely that the inhibitor

functions by neutralizing the positively charged groups or preventing platelet contact with these groups. In view of the selectivity towards cationic groups it is suggested that the binding site(s) on the inhibitor are anionic in nature. The neutralizing effect of the inhibitor on connective tissue is consistent with Zucker and Borrelli's (10) thesis that collagen is the active constituent of connective tissue.

The nature of the inhibitor has been only partially determined. It does not appear to be a lipoprotein and migrates with the alpha globulins at pH 8.6. The activity is very unstable at acid pH and is completely destroyed by trypsin. The results of the ultrafiltration studies suggested a molecular weight greater than 100,000. The sucrose density gradient ultracentrifugation studies were in accord with a molecular weight in the range of 330,000. In some experiments when partially purified fractions were ultracentrifuged on a sucrose density gradient slight inhibitory activity was also found in the lowermost fraction, but this did not occur with serum ultracentrifugation. When the fractions were frozen and thawed and retested the activity disappeared from the lowermost fraction but was retained in the middle fraction on the gradient. This observation may signify the presence of an additional inhibitory activity of greater molecular size (macroglobulin) which is unstable on freeze-thawing or of reversible polymerization of the inhibitor.

A question arises as to the physiological effect of the inhibitor since in most of the experiments collagen and platelet-free inhibitor were incubated before adding platelet-rich plasma and the opportunity for such preincubation may not exist in vivo. The results of the experiment shown in Fig. 5 indicated that varying quantities of inhibitor mixed with platelet-rich plasma can exert a profound influence on the reaction of collagen with platelets without preincubation. It is therefore conceivable that in vivo an increased plasma inhibitor concentration could inhibit platelet plug formation and so impair hemostasis. Such a phenomenon has been described in a case of IgA myeloma in which a clinical bleeding tendency was ascribed to an increased concentration of the IgA immunoglobulin which inhibited connective tissue-platelet interaction (14). Conversely it is possible that a lowered inhibitor concentration could favor the development of platelet aggregates in blood exposed to cationic inducers of platelet adhesion and aggregation.

If microfibrils and other materials in vessel walls which may react with platelets depend for activity on free anionic groups then plasma proteins which neutralize anionic groups may be important in regulating platelet reactivity. Packham, Evans, Glynn, and Mustard (15), showed that solutions containing albumin inhibited platelet adherence to glass tubes and Merrill et al. (16), and Vroman[3] found that albumin inhibited platelet adherence to heparin-coated surfaces.

A possible use for inhibitory fractions may be to coat prosthetic materials so as to render them nonthrombogenic. Specific plasma proteins covalently coupled to the materials from which prosthetic devices are made may be a useful method for inhibiting platelet adhesion at least over a limited period.

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