Platelet Clumping Produced by Connective Tissue Suspensions and by Collagen.* (27335)

MARJORIE B. ZUCKER AND JENNIE BORRELLI
Division of Experimental Surgery and Physiology, Sloan-Kettering Institute for Cancer Research, New York City

Bleeding from small wounds is arrested primarily by masses of blood platelets which literally plug the holes in arterioles and venules(1,2). The platelets are initially discrete but soon seem to fuse so that the plugs appear fairly homogeneous; this process of apparent fusion is known as viscous metamorphosis or VM. Since VM can be produced *in vitro* by thrombin(3,4,5), it has generally been assumed that hemostatic platelet plugs are produced *in vivo* by thrombin formed at the site of vascular damage. However, observations by Hugues(6) on hemostasis in the exposed mesentery of the rabbit demonstrated that the first blood platelets adhered to the margins of a cut blood vessel within 1 or 2 seconds, which is faster than any known mechanism for evolution of thrombin in blood. The Belgian workers also noted that rabbit platelets adhered to strands of connective tissue(7) and to fragments of aorta(8). In attempting to confirm these observations we mixed human citrated platelet-rich plasma with fragments of human tissue obtained at autopsy. Platelets adhered readily to connective tissue but not to muscle or pleural cells. However, we also observed that platelets in the plasma clumped together some distance from the pieces of tissue. Since fibrin did not form, it seemed unlikely that thrombin was responsible. Saline extracts of tissues were then tested and some produced rapid platelet clumping. Some characteristics of the active material and its probable identification as collagen will be described.

Methods. To prepare extracts, about 200 mg of tissue were hand homogenized in a glass tissue grinder with 3 ml 0.9% NaCl. After centrifuging for 5 minutes at about 2500 RPM in a clinical angle centrifuge, sedimented material and supernatant fat were discarded, leaving fairly turbid extracts. Unless otherwise indicated, the tissue was human subcutaneous fat, obtained during radical mastectomy, trimmed free of blood and breast tissue and kept at –20°C. Extracts were also stored at this temperature and could be frozen and thawed several times without losing much activity.

Human platelet-rich plasma was prepared daily in siliconed glassware by slowly centrifuging blood added to 1/9 volume 0.11 M sodium citrate. It was kept at room temperature to avoid cold agglutination of platelets (9). Activity was assayed by adding either tissue extract (usually 0.1 ml) or a dilution of the extract in isotonic saline to an equal volume of platelet-rich plasma. The tubes were gently rocked by hand at room temperature and observed grossly every minute for 3 minutes. The samples were then promptly observed under a phase microscope to detect microscopic clumps and to ascertain whether clumps appeared to be composed of individual platelets or had acquired the fused appearance of VM. In control tests in which platelet-rich plasma was shaken with saline, little or no clumping occurred.

Results. Detection and tissue sources of platelet-clumping activity. Gently shaking citrated platelet-rich plasma with full-strength extracts of subcutaneous connective tissue caused the formation of large clumps within one minute. These usually increased in size within 2 or 3 minutes so that few platelets remained free. Microscopic observation even at one minute revealed clumps in which individual platelets could no longer be discerned (Fig. 1 A). More dilute extracts produced smaller clumps which took longer to become homogeneous in appearance. Potency, measured by determining the highest dilution which produced microscopic clumps in 3 minutes and "fusion" of some areas in 6 minutes, was often 1:100. Unlike Factor R from red blood cells(10), the extracts did not

* This work was supported by a grant from Am. Heart Assn.

increase the adhesion and spreading of platelets on glass slides.

Although the center of clumps produced by strong extracts remained fairly homogeneous, within 30 minutes the margins of the clumps had a faintly radial appearance, and the clumps had shed countless elongated, disc-shaped or rounded fragments of platelets

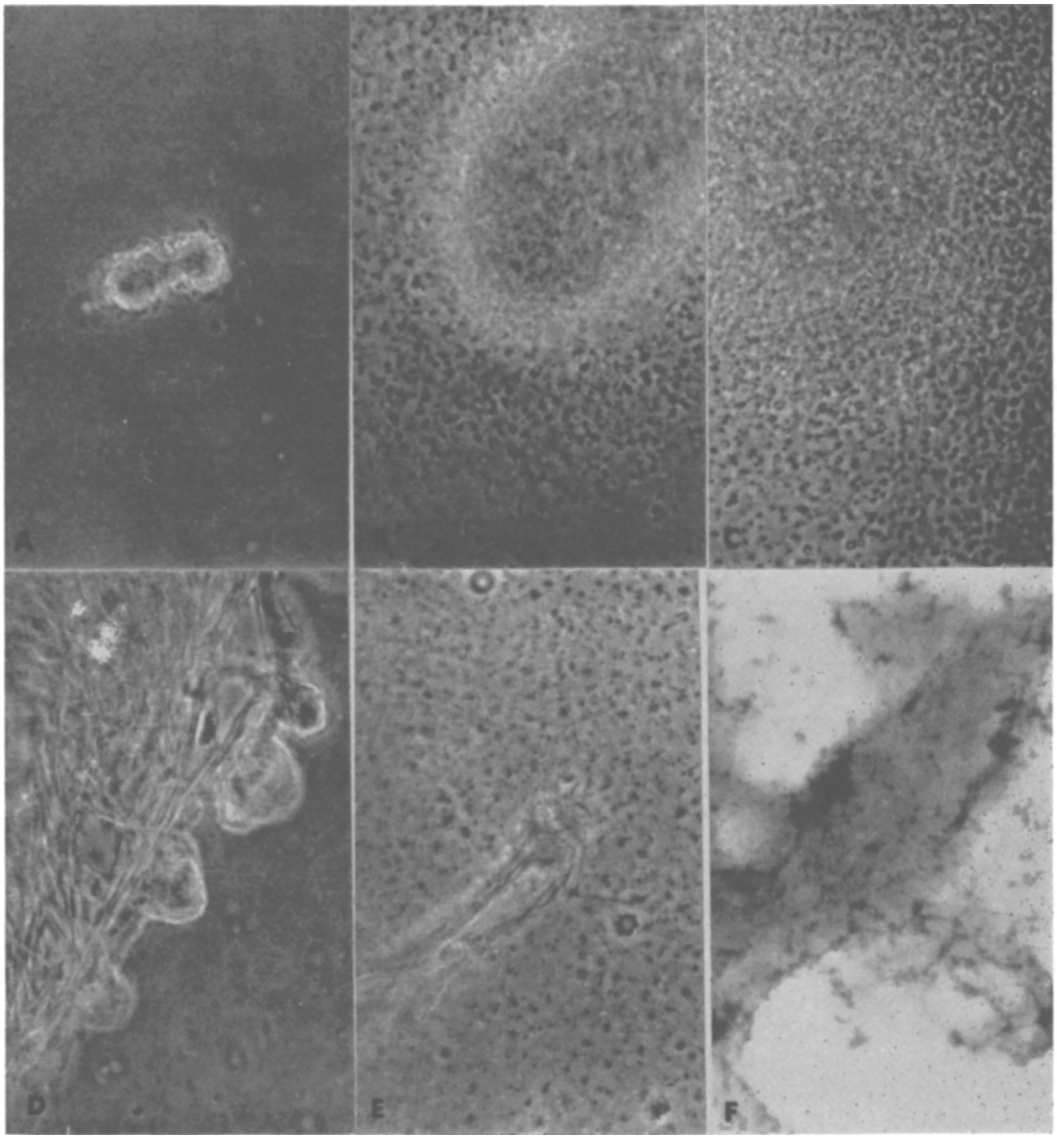

FIG. 1. A. Homogeneous clump 5 min. after shaking human citrated platelet-rich plasma with diluted human subcut. tissue suspension. Phase microscope, 530×. B. Same 35 min. later. Note many free normal and odd shaped platelets and platelet fragments liberated from a former homogeneous clump. C. Same 2 hr later. Core of former homogeneous clump barely visible, surrounded by many liberated platelets and platelet fragments. D. Homogeneous clumps on outer layer of tangled collagen fibers 5 min. after shaking ichthyocol collagen with human citrated platelet-rich plasma. Many single platelets remain in surrounding fluid, and many fibers remain uncoated. E. Same 2 hr later. Remains of homogeneous clump are seen near tip of protruding bundle of collagen fibers. Many free normal and odd shaped platelets and platelet fragments liberated from former homogeneous masses. Larger bodies in surrounding fluid are crenated red blood cells. F. Collagen shaken with platelet-rich plasma and stained 5 min. later with Wright-Giemsa. Darker areas are platelet clumps. No phase, 125×.

which had no conspicuous granules and did not adhere to the glass slide. Tiny free granules and some whole platelets were also seen (Fig. 1 B, C). When diluted extracts were used, apparently intact platelets and a smaller number of fragments gradually separated from the periphery of the clumps, and some clumps dissociated completely. Because of this separation of platelets, we have not used the term "VM" to describe the effect of tissue extracts, even though at first the clumps appeared homogeneous and could not be visually distinguished from those formed by thrombin.

Segments of human aorta were removed at autopsy, frozen, separated into intima, media and adventitia, ground with saline and tested for platelet-clumping activity. All 3 arterial layers were active; the intima had somewhat less activity than the other layers but could still be diluted 100-fold. Tests on an atherosclerotic aorta revealed that an extract of a region of intima which appeared grossly normal could be diluted 100 times, whereas an extract of an atherosclerotic plaque was about half as active and an extract of a calcified area was inactive.

Other tissues were obtained from the rat, mouse and rabbit, and were tested for activity on homologous platelet-rich plasma. Extracts of the following tissues were effective in dilutions of 1:10 or more; subcutaneous fat or connective tissue, skin, aorta, tendon, cornea, sclera, cartilage, vitreous humor, skeletal muscle and sometimes heart muscle. In contrast, spleen, liver, kidney, small intestine and pancreas produced inactive extracts which either failed to produce platelet clumps or produced clumps in which the platelets were loosely packed. Extracts of lung and brain sometimes produced "fused" clumps when used without dilution, but were inactive when diluted 1:10. Extracts of animal tissue were less stable when frozen and thawed than extracts of human subcutaneous tissue. Clumps of rat or rabbit platelets formed by human subcutaneous tissue extracts dissociated more readily on standing than clumps of human platelets.

Chemical characteristics of the connective tissue factor. Full strength extracts, dialyzed against water to remove salt and dried, weighed less than 0.5 mg/ml. Thus less than 0.5 μg could clump many of the platelets in 0.1 ml platelet-rich plasma.

Activity was associated with particulate matter since when extracts of human subcutaneous tissue or rat skin or tail tendon were centrifuged at 25,000 *g* for one hour at 4°C, the supernatant was inactive and activity was recovered by resuspending the sediment in saline. Because of the insolubility of the active factor, extracts will henceforth be called suspensions. No fibers were microscopically visible in the suspensions but some tiny granules were seen. It could not be determined whether or not these granules became incorporated into the platelet clumps. In any event, there were too few visible particles to account for the effect of diluted tissue suspensions. After treatment of saline suspensions with ultrasonic vibrations (Mullard machine, Instrumentation Associates, New York) for 3 minutes, activity was doubled but active material remained insoluble.

In an attempt to dissolve the active material, human subcutaneous tissue was ground with 0.05 M HCl-KCl buffer pH 1.8, 1 M $CaCl_2$ in 0.1 M sodium acetate(11) 0.05 M glycine-NaOH buffer pH 10.6, water, 0.15 M NaCl, 1 M NaCl, or 2 M potassium thiocyanate. The suspensions were submitted to preliminary centrifugation to remove gross debris and fat and were placed at 4°C overnight. After removing a small aliquot, they were then centrifuged for one hour at 25,000 *g* at 4°C. The sediments were resuspended in saline and the sediments, the top layer of supernatant, and the small aliquot of uncentrifuged suspension were dialyzed against saline. About 20% of the total activity was found in the supernatants after extraction of the tissue with the acid buffer or with $CaCl_2$ whereas the remaining extractives dissolved less than 10% of the activity. Both the sediment and whole suspension prepared at pH 1.8 were 2 or 3 times more active than material prepared with the other solutions.

Heat stability at various pHs was investigated. Five minutes at 100°C caused loss of all activity at pH 1.8 and 5.0 and loss of about 80% activity at pH 7.8 and 10.6. At

pH 7.2, 30 minutes at 100°C caused loss of all activity; at 70°C, 80% of the activity was abolished, whereas at 56°C, activity increased 2-fold.

When human subcutaneous tissue was ground with acetone, activity remained in the acetone-insoluble fraction. Activity of saline suspensions was adsorbed by $BaSO_4$ but not eluted with 0.2 M sodium citrate solution. Overnight incubation at 37°C in 1 M sodium nitrite or neutralized 0.1 M hydroxylamine followed by dialysis failed to abolish activity. Incubation at 37°C for one hour with 10 volumes of formamide caused complete destruction, whereas incubation for 10 minutes at room temperature had no effect. Addition of ethanol in a final concentration of 25% followed by evaporation of the ethanol under vacuum at room temperature caused loss of almost all activity; 12.5% ethanol caused some decrease in potency.

A homogenized saline suspension of the precipitate remaining after 2 extractions at −14°C with acetone, followed by one extraction with acetone-dry ether and one with dry ether(12) retained 25 to 50% of the original activity. The lipid soluble fraction, resuspended in saline after evaporation of solvents at low pressure, had no effect on platelets. When lipid was extracted by simply shaking saline suspensions of human subcutaneous tissue with ether at room temperature, activity remained in the water layer in one experiment. On 2 subsequent occasions, activity was lost and a layer of presumably denatured material developed at the interface.

Active suspensions were incubated at 37°C at pH 7.0 to 7.7 for 1 hour with trypsin, chymotrypsin, papain with and without cysteine, testicular hyaluronidase, and elastase, and with pepsin at pH 1.8. Although the enzymes were active when tested on appropriate substrates, they failed to destroy platelet-clumping activity. Active material remained sedimentable after heating to 56°C for 30 minutes and subsequent overnight incubation with trypsin. It was not inhibited by diisopropyl-phosphofluoridate (DFP) or soy bean trypsin inhibitor in concentrations greater than those which respectively inhibited clotting produced by thrombin or trypsin.

The sodium salts of hyaluronic acid, chondroitin sulfate A, keratosulfate and heparitin sulfate, 0.5 mg/ml in veronal buffer pH 7, failed to clump platelets in an equal volume of platelet-rich plasma. Chondroitin sulfates B and C produced small clumps after 2 minutes of shaking. Mixing connective tissue suspension with the polysaccharides 3 minutes before testing did not inhibit the clumping produced by the connective tissue.

Comparison of connective tissue factor with collagen. Initial experiments with commercial collagenase appeared to indicate that this enzyme did not destroy activity of tissue suspensions. However, it was not appreciated that collagenase required calcium ions and hence was probably inactive in the presence of phosphate buffer. Furthermore, the enzyme preparations contained other proteolytic enzymes.

Subsequent studies were made with highly purified collagenase(11) which was kindly provided by Dr. S. Seifter of Albert Einstein College of Medicine. Collagenase alone had no effect on platelets but abolished platelet-clumping activity of saline suspensions of human subcutaneous tissue or mouse tail tendon in the presence of 0.001 M calcium chloride.

The ability of collagenase to destroy activity of connective tissue suspensions led us to test the effect on platelets of purified collagen obtained from calf skin or fish swim bladders (ichthyocol)(12) which was also supplied by Dr. Seifter. Collagen fibers were suspended in saline solution and the collagen suspension shaken with an equal volume of platelet-rich plasma. Most of the platelets disappeared from the plasma rather suddenly after 1 to 2 minutes of shaking. They were found in homogeneous clumps, most of which were attached to the collagen fibers. Some fibers were entirely coated with masses of "fused" platelets, others had a few clumps attached at irregular intervals, and still other fibers, equally exposed, were completely free of adhering platelets. Similar clumps of "fused" platelets were also found unattached to visible collagen fibers. During the ensuing hour or two, both free and attached clumps

underwent the same morphologic changes as did clumps formed by strong extracts of subcutaneous tissue (Fig. 1 D, E and F).

When a suspension of collagen was ground in the same manner as whole tissue and centrifuged at about 1000 RPM for a few minutes, the supernatant had no effect upon the platelets, demonstrating that, unlike suspensions of subcutaneous tissue, all of the active material was very easily sedimented. Gelatin, formed by heating the collagen suspension to 56°C for 30 minutes was inactive on platelets, as were neutral solutions of proline, hydroxyproline, glycine and tyrosine.

Inhibitor in liver. Since extracts of parenchymatous organs do not clump platelets even though they contain connective tissue, Dr. T. H. Spaet suggested that these organs might contain an inhibitor. To test this hypothesis, saline extracts of rat liver were prepared in the same manner as other tissue extracts. Between one-tenth and 1 volume of liver extract was mixed with 1 volume of suspension of rat skin, tail tendon or subcutaneous tissue, diluted suspension of human subcutaneous tissue, or purified collagen. Provided the liver extract was incubated with the connective tissue suspensions for 2 minutes at room temperature before testing the mixture on human or rat platelet-rich plasma, very little or no platelet clumping occurred. The amount of rat liver extract required for complete inhibition depended on the potency of the connective tissue suspension employed. The inhibitor was stable at 4°C overnight and at –20°C for at least several months. It was not dialyzable, and was inactivated by boiling for 5 minutes. When rat liver extract was centrifuged at 25,000 *g* for 30 minutes, the inhibitor was recovered in the supernatant and not in the sediment. Whether the inhibitor destroys the active material or blocks its effect is not yet known.

Dissociation of platelet-clumping activity from coagulation. Suspensions of human subcutaneous tissue did not contain detectable thrombin since they did not clot plasma or fibrinogen solutions in 18 hours at 37°C. They had weak thromboplastic activity which was about equal to a 1:50 dilution of acetone-dried human brain thromboplastin when tested on plasma from normal subjects or patients with severe congenital deficiencies of antihemophilic factor (AHF or factor VIII), plasma thromboplastin component (PTC or factor IX), or plasma thromboplastin antecedent (PTA). It seemed unlikely that the thromboplastic activity was responsible for the effect of tissue suspensions on platelets because 1:50 or undiluted human or rabbit brain thromboplastin either failed to clump platelets in citrated plasma or caused only loose clumps. To provide more direct evidence and to enable us to test suspensions on native platelet-rich plasma, thromboplastic activity of suspensions of subcutaneous tissue, measured by clotting time of recalcified plasma, was almost completely abolished by incubation with 2 mg of bacterial thromboplastinase per ml(13).† After this treatment the effect of the suspensions on platelets in citrated platelet-rich plasma was unchanged although the activity was no longer stable when the suspensions were frozen. Thromboplastic activity was also much reduced by treating suspensions with acetone and ether at –14°C. As previously stated, this reduced the platelet-clumping activity 50 to 75% when it was tested on citrated platelet-rich plasma, but considerable activity remained, and suspensions were stable when frozen.

Representative tests on native plasma are shown in Table I. When platelet-rich plasma was shaken with saline, platelet clumping did not occur for 20 minutes and was quickly followed by coagulation. This supports our earlier conclusion that thrombin causes platelet aggregation in native platelet-rich plasma (9). Untreated tissue suspensions caused platelet clumping in less than a minute, and this was immediately succeeded by clotting. This rapid effect on platelets might be produced by the tissue factor itself, or by thrombin, evolved rapidly because of the thromboplastic activity of the suspensions. When suspensions poor in thromboplastic activity were used, platelets clumped as rapidly as with untreated suspensions, but clotting occurred much more slowly, suggesting that the

† The thromboplastinase was kindly given us by Dr. S. Gollub, Hahnemann Medical College.

TABLE I. Effects of Connective Tissue Suspensions with Diminished Thromboplastic Activity and Collagen on Platelet Clumping Time and Clotting Time.

		Native plat-poor pl.		Native plat-rich pl.			
		Glass	Silicone	Glass		Silicone	
Exp.	Test material*	Clot time	Clot time	Clump time	Clot time	Clump time	Clot time
1	Saline		27			20	20½
	Susp.		1			½	1
	Susp. + T'ase.†		19½			½	4¼
	Saline + T'ase.†		18			7½	8¼
2	Saline	8	25			20	21
	Susp.	1½	1½			½	1½
	Lipid-free susp.	12	11			½	4
3							
PTA‡	Saline			23; > 30	26; 40	> 30; > 30	92; > 80
	Susp.					½	1½
	Susp. + T'ase†					¼	> 30
	Collagen			1¼; 1½	13; 24	1¼; 2	70; 45
4	Saline + saline			3½	4½		
	Susp. + saline			1	2		
	Heparin§ + saline			> 30	> 30		
	Heparin§ + susp.			1	> 30		
5	Saline	4	8½	3	3½	20	22
	Collagen	4	7	1¾	3½	1¾	6
6	Saline	4½	11	3½	4	12	14
	Collagen	4	9½	1¼	4	1½	6

* 0.1 ml test material shaken with 0.25 ml native platelet-rich or platelet-poor plasma. Clumping and clotting times are given in minutes.

† Suspension incubated with thromboplastinase for 1 hr at 37°C before use.

‡ Blood was obtained through the courtesy of Dr. Robert Rosenthal from the 2 patients in whom PTA deficiency was originally described(23).

§ Final heparin concentration in plasma was 5 units/ml.

suspensions affected platelets independently of coagulation. This conclusion was supported by the results on plasma from patients with severe PTA deficiency (Exp. 3). The effects of tissue suspensions on platelets and coagulation were also distinguished by use of heparin. When suspensions were tested on heparinized platelet-rich plasma, platelet clumps formed as rapidly as usual, but gross clotting and even microscopic fibrin formation were prevented for more than 30 minutes (Exp. 4). When as much as 50 units heparin were added to 1 ml citrated platelet-rich plasma, the effect of undiluted suspensions was undiminished, and the effect of a 1:100 dilution was only slightly decreased. Preincubation of heparin with suspensions also failed to produce significant inhibition of platelet clumping.

Since even the tissue suspensions poor in thromboplastic activity caused some shortening of the clotting time of native platelet-rich or platelet-poor plasma, it seemed worthwhile to determine whether collagen itself affected the clotting time. As shown in Exp. 5 and 6, addition of 0.05 mg collagen shortened the clotting time of native platelet-rich and platelet-poor plasma in siliconed tubes. Clotting time of recalcified citrated platelet-poor plasma was slightly shortened in both glass and siliconed tubes. When phospholipid was incorporated, however, collagen failed to shorten the clotting time of recalcified citrated platelet-poor plasma in either glass or siliconed tubes. Collagen thus seems to behave like very weak thromboplastin rather than like glass or kaolin. Extraction of collagen with lipid solvents(12) did not alter this action.

Washed or frozen platelets, like cephalin, shorten the plasma Stypven time (Russell Viper Venom, Burroughs Wellcome Co.) from about 24 seconds to about 7 seconds, whereas platelets in platelet-rich plasma are ineffective(14). Stypven times were not shortened when platelets were clumped a few minutes or an hour earlier by shaking platelet-rich plasma with connective tissue suspensions (Table II). It was necessary to use either dilute or lipid-poor suspensions for these tests

TABLE II. Effect of Platelet Clumping on Stypven Time of Platelet-Rich Plasma.

Exp.	Citrated plasma	Test material	Occurrence of platelet clumping	Stypven time seconds
1	Plat-poor	1:50 susp.	—	22.4
	Plat-rich	" "	Yes	25.8
	"	Saline	No	25.2
2	Plat-poor	Lipid-free susp.	—	24.5
	Plat-rich	" "	Yes	25.2
	"	Saline	No	23.5
3	Plat-poor	"	—	26.0
	"	Collagen	—	22.2
	Plat-rich	Saline	No	25.4
	"	Collagen	Yes	19.6

0.2 ml plasma + 0.2 ml test material shaken for 5 min. An aliquot is observed microscopically, and 0.2 ml is added to 0.1 ml Stypven + 0.1 ml .0125 M $CaCl_2$.

since undiluted suspensions alone shortened the Stypven time. Thus the tissue factor did not make platelet cephalin "available". Collagen slightly shortened the Stypven time of both platelet-rich and platelet-poor plasma.

Studies on patients with hemorrhagic diatheses. Suspensions did not clump the platelets in citrated platelet-rich plasma from 2 patients with thrombasthenia.‡ Platelets from 3 patients with pseudohemophilia§ (von Willebrand's disease) or congenital deficiencies of AHF, PTC and PTA reacted normally.

Suspensions were made from skin and subcutaneous tissue from the first 2 patients with pseudohemophilia (see footnote). At the time of biopsy,‖ the first patient was bleeding from a colostomy and had several spontaneous ecchymoses. The second patient bled from his wound an hour after biopsy even though numerous sutures had been used. The tissue suspensions clumped platelets in normal or the patients' platelet-rich plasma in dilutions up to 1:150 to 1:500, which was within the range of activity of samples of skin and subcutaneous tissue obtained during operations from individuals with normal hemostasis.

Comparison of effects of connective tissue suspension and thrombin on platelets. Platelet clumping produced by thrombin is inhibited by mercuric ions and by antihistaminic drugs such as benadryl which cause platelet sphering(3). Platelet clumping produced by suspensions was similarly inhibited in the presence of 10^{-3} M $HgCl_2$ or 1 mg/ml benadryl. Platelet-rich plasma from blood collected with 1/9 volume 1% (0.027 M) disodium ethylenediaminetetraacetate (EDTA) in saline failed to react with connective tissue factor unless 0.013 ml 0.04 M $MgCl_2$ was added to 0.1 ml EDTA platelet-rich plasma. Clot retraction and platelet clumping produced by thrombin are also inhibited by EDTA and restored by this concentration of magnesium(15).

Platelets separated from EDTA blood by differential centrifugation, washed 3 times with isotonic saline, and suspended in .05 M sodium phosphate buffer of pH 7.75 or 7.0 or imidazole buffer pH 7.3 underwent marked VM with human thrombin(3) but formed only a few loose clumps with collagen or strong connective tissue suspensions. Even when the washed platelets were first incubated at room temperature in citrated platelet-poor plasma, addition of suspensions was relatively ineffective. When thrombin is added to citrated platelet-rich plasma or to washed platelets suspended in buffer, about 50% of platelet adenosine triphosphate (ATP) disappears within 15 minutes(16). In contrast, platelet clumps produced in

‡ These patients were not related to each other. Both had a history of bleeding, long bleeding times and consistently showed severely impaired clot retraction despite normal platelet counts. Their platelets failed to show VM when either native platelet-rich plasma(9) or washed platelets plus thrombin(3) was tested. Both had moderately abnormal prothrombin consumption which was attributed to a platelet defect since clotting factors in plasma and serum were normal as judged from thromboplastin generation test.

§ These unrelated patients had significant histories of excessive bleeding. One patient had a positive family history, repeated bleeding times (Ivy) of about 20 min. and normal AHG on several occasions; the second had a positive family history, subnormal AHG (29%) and a consistently prolonged bleeding time; and the third had less than 40% AHG on one occasion, and 64 and 89% on other occasions, and a bleeding time of 9 min. on 2 occasions.

‖ We are greatly indebted to Dr. E. E. Cliffton for performing the operations, and to our patients for their amiable cooperation.

platelet-rich plasma by connective tissue factor lost no ATP in 15 minutes.

Discussion. Both saline suspensions of ground connective tissue and purified collagen clump the platelets in citrated platelet-rich plasma. The active material in connective tissue, like collagen, is destroyed by highly purified collagenase. This enzyme has considerable specificity, affecting, besides collagen, only mixed polymers of proline and glycine(11). The platelets in clumps formed by tissue suspensions or by collagen undergo morphologically similar packing and fragmentation. Like collagen, the active material in connective tissue is not soluble in saline and is partially soluble in acid or in 1 M $CaCl_2$ in 0.1 M sodium acetate(11). However, unlike purified collagen, it is stable at 56°C. Furthermore, when collagen is ground in isotonic saline the active material is sedimented much more easily than the active substance in connective tissue. It seems likely that the connective tissue material is either a combination of collagen with some other tissue constituent or a compound with proline-glycine bonds like those of collagen. If it proves to be distinct from collagen, we propose it be called thrombadhesin.

Hugues and Bounameaux have described adhesion of platelets to connective tissue fibers(7) and fragments of aorta(8), and Hugues recently reported (8th European Congress of Hematology, Vienna 1961) that platelets adhere to purified collagen fibers. We are probably studying the same phenomena as they are, but our findings differ in certain respects. For example, they have not observed platelet aggregation independent of fibers, and they find that washed platelets are effective. Some of the discrepancies may be ascribed to species differences since they used rabbit tissue and platelets rather than material of human origin.

The term VM, ordinarily used to describe the effect of thrombin on platelets, was used at first to describe the effect of connective tissue suspensions and collagen as well, because the clumps appeared under the phase microscope to be composed of platelets which had lost their individuality and fused together. There are other similarities, too, between the effects of tissue suspensions and thrombin; both fail to clump platelets from patients with thrombasthenia, and are ineffective against normal platelets in the presence of mercuric chloride, benadryl or EDTA. On the other hand, thrombin produces a fall in platelet ATP(16,17), and probably also exposes platelet phospholipid so that platelets can play their role in prothrombin consumption, whereas connective tissue suspensions are inactive in these respects. Using the latter criteria rather than morphological appearance, connective tissue suspensions do not produce VM.

We suggest that the connective tissue substance causes the initial adhesion of platelets to a cut blood vessel and to each other during hemostasis. Other possible causes are adenosine diphosphate or Factor R from red blood cells(18,19), and endothelial cells which become sticky when injured(20). Connective tissue probably only comes in contact with the blood and causes local platelet aggregation when a blood vessel is cut or endothelium is damaged. This may be important not only in hemostasis, but also in thrombosis and even atherosclerosis. Perhaps the paucity of connective tissue in the walls of capillaries accounts for the observation that hemostasis in the true capillaries of the mesoappendix, skin and liver is not accomplished by platelet plugs but by adhesion of the endothelium or by a fibrin clot(1,2, 21). Perhaps, too, the excessive bleeding from the abnormal dilated capillaries in the lesions of hereditary hemorrhagic telangiectasis can be explained on this basis. The platelets of patients with pseudohemophilia react normally with connective tissue and suspensions of subcutaneous tissue from 2 patients had normal activity. The nature of the abnormality responsible for the prolonged cutaneous bleeding time in this congenital disorder still eludes us. Possibly there is a localized deficiency of connective tissue factor in tissue surrounding the blood vessels in these subjects which might, in addition, explain the abnormal tortuosity of the microscopic blood vessels(22).

Summary. Saline suspensions of a variety of connective tissues and of collagen clump

platelets in citrated platelet-rich plasma even in dilutions of 1:100. The effect is unrelated to blood clotting. The active material in human subcutaneous tissue is sedimented at 25,000 *g* in one hour, stable at 56°C, but not at 70°C, and destroyed by collagenase, formamide and 25% alcohol but not by proteolytic enzymes. An inhibitor is present in liver. Similarities and differences between the effects of purified collagen, tissue suspensions and thrombin on platelets are described and the probable role of the connective tissue factor in hemostasis is discussed.

1. Zucker, M. B., *Am. J. Physiol.*, 1947, v148, 275.
2. Zucker, H. D., *Blood*, 1949, v4, 631.
3. Zucker, M. B., Borrelli, J., *J. Appl. Physiol.*, 1959, v14, 575.
4. Lüscher, E. F., *Vox Sang.*, 1956, v1, 133.
5. Bounameaux, Y., *Rév. hemat.*, 1957, v12, 16.
6. Hugues, J., *Thrombosis Diath. Haem.*, 1959, v3, 177.
7. ———, *C. R. Soc. Biol.*, 1960, v154, 866.
8. Bounameaux, Y., *Thrombosis Diath. Haem.*, 1961, v6, 504.
9. Zucker, M. B., Borrelli, J., *ibid.*, 1960, v4, 424.
10. Hellem, A. J., *Scand. J. Clin. Lab. Invest.*, 1960, Suppl. 51, v12.
11. Seifter, S., Gallop, P. M., Klein, L., Meilman, E., *J. Biol. Chem.*, 1959, v234, 285.
12. Blix, G., *ibid.*, 1941, v137, 495.
13. Gollub, S., *J. Appl. Physiol.*, 1955, v7, 409.
14. O'Brien, J. R., *J. Clin. Path.*, 1959, v12, 45.
15. Zucker, M. B., Borrelli, J., *J. Appl. Physiol.*, 1958, v12, 453.
16. ———, in *Blood Platelets*, Ed., Johnson, S. A., Monto, R. W., Rebuck, J. W., Horn, R. C., Jr., Little, Brown & Co., Boston, 1961, p. 383.
17. Bettex-Galland, M., Lüscher, E. F., *Thrombosis Diath. Haem.*, 1960, v4, 178.
18. Gaarder, A., Jonsen, J., Laland, S., Hellem, A., Owen, P. A., *Nature*, 1961, v192, 531.
19. Hellem, A. J., Borchgrevink, C. F., Ames, S. B., *Brit. J. Hematol.*, 1961, v7, 42.
20. O'Brien, J. R., *J. Clin. Path.*, 1961, v14, 140.
21. Apitz, K., *Z. ges. exp. Med.*, 1942, v111, 554.
22. Schulman, I., Smith, C. H., Erlandson, M., Fort, E., Lee, R. E., *Pediatrics*, 1956, v18, 347.
23. Rosenthal, R. L., Dreskin, O. H., Rosenthal, N., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 171.

Received September 14, 1961. P.S.E.B.M., 1962, v109.

Kinetics of Plasma Hemoglobin Catabolism in the Rat. I. Blood Clearance of I^{131} Tagged Hemoglobin.* (27336)

E. R. GABRIELI, P. HECKERT, AND A. ELLIOTT
Millard Fillmore Research Institute, Buffalo, N. Y.

Despite a vast accumulation of experimental data, the precise mechanism by which circulating hemoglobin is cleared from the blood is still undetermined. Whipple's classic theory of a "renal threshold" claimed that excretion by the kidneys occurred only when the plasma hemoglobin level exceeded this "threshold". Laurell and Nyman(1) subsequently hypothesized that in humans the hemoglobin binding capacity of the plasma haptoglobin is actually the "renal threshold". Hemoglobin, combined with haptoglobin, they maintained, is removed and degraded, presumably by the reticulo-endothelial system, while unbound hemoglobin in excess of haptoglobin is rapidly excreted by the kidneys. This appealingly simple concept, although confirmed in humans by Lathem(2), was not apparent when the clearance of injected hemoglobin was investigated in laboratory animals. In rabbits, Murray *et al.* (3) suspected the liver to be the site of degradation of the hemoglobin-haptoglobin complex. No "free" hemoglobin appeared in the urine even when the injected hemoglobin exceeded the haptoglobin level. However, this is at variance with the findings of Franklin and his associates(4) who were unable to demonstrate either reticulo-endothelial participation or preferential hepatic removal of

* This work was supported by Grant from Nat. Inst. of Health.