Plasmin-Induced Platelet Aggregation and Platelet Release Reaction

EFFECTS ON HEMOSTASIS

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Abstract Trypsin-activated pig plasmin and human plasmin activated by streptokinase (SK) caused aggregation of a suspension of washed platelets from human, rabbit, or pig blood. The platelet aggregation was reversible, but it was accompanied by a significant release of adenine nucleotides, serotonin, and platelet fibrinogen. Platelet fibrinogen was eventually digested. The effect of plasmin on platelets was inhibited by soybean trypsin inhibitor, epsilon aminocaproic acid, Persantin, prostaglandin E_1, and phenylbutazone. Short treatment of platelets with plasmin enhanced their sensitivity to ADP; however, this sensitivity was lost during longer incubation with plasmin. This enzyme also made platelets less sensitive to collagen and thrombin.

Injecting SK into rabbits (10,000 U/kg body weight) caused a transitory drop of platelet count. These platelets lost part of their serotonin and fibrinogen. The administration of Persantin or of epsilon aminocaproic acid to rabbits before the injection of SK protected platelets from the loss of serotonin. Pretreatment with Persantin also resulted in partial protection of platelet fibrinogen in rabbits injected with SK. Platelets obtained from rabbits that had received both Persantin and SK were much more reactive with collagen than platelets obtained from rabbits injected with SK alone. Rabbits pretreated with Persantin did not show prolongation of the primary bleeding time that occurred after SK injection to control rabbits. It is suggested that plasmin generated after SK injection causes platelet release reaction in vivo. This may contribute to the hemostatic defect occurring during thrombolytic therapy or during systemic activation of fibrinolysis due to the other factors.

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INTRODUCTION

It is well established that platelet aggregation and the platelet release reaction can be brought about by several proteolytic enzymes such as thrombin (1) papain, trypsin, and pronase (2). Niewiarowski, Gurewich, Senyi, and Mustard have recently reported that plasmin causes aggregation of suspensions of washed human, pig, and rabbit platelets. This aggregation was accompanied by the release of serotonin (3). The purpose of this work is to investigate further the effects of plasmin on platelet function in vitro as well as the effects of the infusion of streptokinase (SK)[1] which leads to the activation of fibrinolytic system in vivo. It was found that plasmin causes reversible aggregation of platelets accompanied by a significant release of adenine nucleotides, serotonin, and platelet fibrinogen which is subsequently digested. The release of platelet constituents and reversible platelet aggregation also occurred in vivo after injection of SK. We present evidence that these phenomena may contribute to the hemostatic defect in primary fibrinolysis.

METHODS

Isolation of platelets. Washed human, pig, or rabbit platelets prepared according to the method of Ardlie, Pack-

[1] *Abbreviations used in this paper:* ACD, acid citrate-dextrose solution; DFP, diisopropylfluorophosphate; EACA, epsilon aminocaproic acid; 5-HT, 5-hydroxytryptamine; LDH, lactic dehydrogenase; LTU, light transmission units; PGE_1, prostaglandin E_1; SBTI, soybean trypsin inhibitor; SK, streptokinase.

ham, and Mustard, (4) or Mustard, Perry, Ardlie, and Packham (5) were suspended in Tyrode-albumin solution containing apyrase. The platelet count of the final suspension was 10^9/ml, if not otherwise stated. In some experiments platelet-rich citrate plasma (pH 7.4) was used instead of the suspension of washed platelets.

Platelet aggregation. Aggregation of platelets were studied by the method of Mustard, Hegardt, Rowsell, and MacMillan, (6) using a Payton Aggregometer (Payton Associates, Inc., Scarborough, Ontario). Results were expressed in arbitrary units (light transmission units, LTU), which correspond to the light transmission change.

Aggregation stimuli. ADP (Sigma Chemical Co., St. Louis, Mo.), collagen (Sigma), and thrombin (Parke, Davis & Co., Detroit, Mich.) were used as aggregation stimuli. Collagen suspension was prepared by homogenizing 10 g of collagen in 100 ml of Tyrode's solution for 20 min in a Waring Blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.). The coarse particulate material was removed by centrifugation at 3000 *g* for 10 min at 4°C. Acid-soluble collagen was a gift of Dr. J. F. Mustard.

Labeling platelets with [^{3}H]serotonin([^{3}H]5-hydroxytryptamine([^{3}H]5-HT)) and measuring the in vitro release of radioactivity from platelets labeled with [^{3}H]5-HT. These assays were performed as described previously (7). [^{3}H]5-HT (Amersham/Searle Corp., Arlington Heights, Ill.) had specific activity of 5.4 mCi/mg.

Adenine nucleotide release from platelet suspension was measured as described previously (7).

Lactic dehydrogenase (LDH) assay. This was done using the method of Bergmeyer, Bernt, and Hess (8). Released activities were expressed as percentages of the total activity of platelet suspension.

Platelet fibrinogen. The staphylococcal clumping test of Hawiger, Niewiarowski, Gurewich, and Thomas (9) has been modified to estimate the content of fibrinogen in platelets. The powdered preparation of staphylococci containing clumping factor from the culture of *Staphylococcus aureus,* Newton D_2C strain was supplied by Sigma. Bacteria were

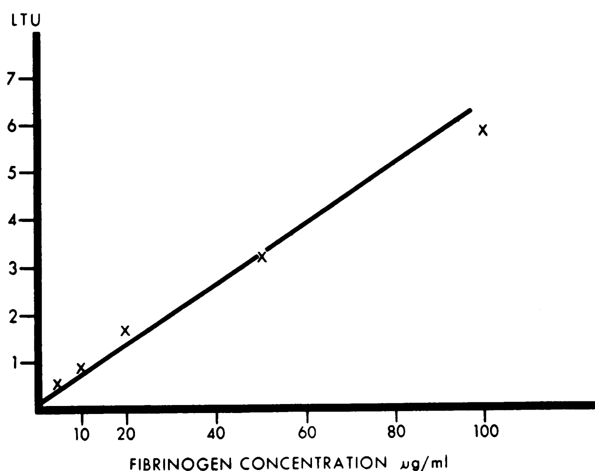

FIGURE 1 Effect of fibrinogen concentration on the light transmission changes of the staphylococcal suspension. 1 ml of 0.1% staphylococcal suspension was incubated for 3 min with 0.1 ml of fibrinogen in a Payton aggregometer and light transmission changes were recorded automatically.

resuspended in Tris buffer of pH 7.4 and adjusted to the final concentration of 0.1% of dry weight. 1-ml samples were pipetted to the aggregometer cuvettes. Standard solution of human fibrinogen (Kabi, Stockholm, Sweden; grade L, 90% clottable protein) or 95% clottable rabbit fibrinogen (kindly supplied by Dr. E. Regoeczi) was diluted in Tris buffer. 0.2 ml of fibrinogen at the concentration ranges of 2–200 µg/ml were added to the samples of bacterial suspension and the increase of light transmission was recorded in a Payton Aggregometer. Fig. 1 shows a correlation between the concentration of fibrinogen and the increase of light transmission during a 3-min incubation period of bacteria with fibrinogen. There was virtually no difference between the reactivity of human and rabbit fibrinogen. Human fibrinogen was used in most experiments for the preparation of the calibration curve. A suspension of platelets (10^9/ml), cooled in the ice bath, was sonicated for 1 min in Virsonic Cell Disrupter (The Virtis Co., Inc., Gardiner, N. Y.). 0.2-ml samples of sonicated platelets were added to the bacterial suspension and the light transmission change was recorded. The level of fibrinogen was calculated by intrapolation from the standard curve (Fig. 1).

Plasmin. Trypsin-activated pig plasmin (Novo-Fabrik, Copenhagen) contained 2.2 Remmert and Cohen casein U/mg. In some experiments, human plasminogen (kindly supplied by Dr. A. J. Johnson, American National Red Cross) activated by SK (Streptase, gift of Hoechst Pharmaceuticals, Canada, Ltd.) was used instead of plasmin. 0.1 ml of 0.25% plasminogen solution (containing 14.4 CTA U/mg protein) was incubated with 0.1 ml of SK (500 U/ml) for 3 min before addition to the platelet suspension.

The enzymes were tested for the possible contamination by thrombin or trypsin. 0.1 ml of plasmin (or of plasminogen) was mixed with 0.1 ml of 0.5% soybean trypsin inhibitor (SBTI, Sigma). After 5 min of incubation the mixture was added to 0.2 ml of 0.5% human fibrinogen and incubated further for 3–24 h at room temperature. Since SBTI does not inhibit thrombin but does inhibit plasmin, the absence of fibrin and the presence of clottable protein in the incubation mixture indicated the lack of any appreciable contamination by thrombin. To evaluate a possible contamination of pig plasmin by trypsin, the heat stability of both enzymes was compared. The fibrinogenolytic activity of plasmin and of trypsin (Sigma) was measured by incubating 0.1 ml of enzyme (serial dilutions) and 0.4 ml of 0.5% human fibrinogen for 5 min. Then the presence of clottable protein in the mixture was estimated by the addition of 0.1 ml of thrombin (100 U/ml). The fibrinogenolytic titer of 0.5% plasmin and of 0.05% trypsin amounted to 256. After incubation of enzyme solutions at 80°C and at pH 4.0 for 10 min the fibrinogenolytic titer of trypsin remained unchanged while plasmin became completely inactivated. Other experiments also demonstrated that incubation at 80°C does not affect the ability of trypsin to aggregate platelets, but it destroys plasmin effects on platelets. These experiments indicate absence of any appreciable amount of residual trypsin in the trypsin-activated pig plasmin.

Incubation of platelets with plasmin in vitro. 0.1 ml plasmin solution in 0.9% NaCl was added to 1.0 ml of plateet suspension and incubated either in aggregometer cuvettes or in the test tubes kept in a water bath at 37°C for various time intervals. Although the plasmin solution was acidic, the pH of the mixture of plasmin and platelet suspension amounted to 7.4. In other experiments, 0.2 ml of the mixture of human plasminogen and SK was added to 1.0 ml of platelet suspension. Changes in light transmission occur-

ring during platelet incubation with plasmin were measured by a Payton Aggregometer. The action of plasmin on platelets was stopped by addition of 0.1 ml of 0.5% SBTI. In the release study, immediately after aggregation, platelets were centrifuged for 1 min at 15,000 *g* (Eppendorf centrifuge, Brinkmann Instruments, Rexdale, Ontario). [^{3}H] 5-HT radioactivity, adenine nucleotides, and lactic dehydrogenase (LDH) were measured both in the sediment and in the supernate after release. The addition of pig plasmin (at the final concentration of 0.02–0.05%) to the platelet-suspending fluid (Tyrode-albumin solution) containing no platelets resulted in the formation of microprecipitate. The changes of light transmission related to this phenomenon were recorded with the Payton aggregometer. First a decrease of light transmission simulating change of platelet shape, and then a small increase of light transmission simulating slight platelet aggregation were recorded. It has been found that the addition of lysine hydrochloride (at the final concentration of 0.02 M) to the platelet-suspending medium prevented the precipitation of plasmin at pH 7.4. The addition of 0.1 ml of 0.5% plasmin to 1.0 ml of suspending medium containing lysine did not result in any change of light transmission.

Inhibitors of the plasmin action on platelets. SBTI and epsilon aminocaproic acid (EACA, Calbiochem, San Diego, Calif.) were dissolved in 0.9% NaCl. Phenylbutazone (gift of Geigy Pharmaceuticals, Ltd., Montreal) dissolved in distilled water and solubilized with 1 N NaOH (pH 7.3). Persantin (dipyridamole, gift of Boeringer Ingelheim, Canada, Ltd.) was diluted to a desired concentration with Tyrode's solution. Solution of prostaglandin E_1 (PGE_1) gift of Upjohn Co., Kalamazoo, Mich.) was made up as previously described (7). SBTI and EACA inhibited fibrinolytic activity of plasmin, but Persantin and PGE_1 did not affect fibrinolysis.

Activation of fibrinolysis in vivo. SK was dissolved in sterile saline or in Tyrode solution to a concentration of 50,000 U/ml and was injected into the ear veins of rabbits in a dose of 10,000 U/kg body weight. Control rabbits were infused with the equivalent volume of sterile Tyrode or saline solution.

The following measurements were made to evaluate the extent of the activation of fibrinolysis: euglobulin lysis time (10), thrombin time of platelet-poor plasma, and the lysis time of clots formed from the whole blood. Fibrinogen level in plasma was measured as a clottable protein by the method of Astrup, Brakman, and Nissen (11) or by the staphylococcal clumping assay. For the latter test, plasma was diluted 1:100 with Tris buffer (pH 7.4).

Platelet count was measured either by the phase-contrast microscopy or by electronic counting (Electrozone celloscope, Particle Data, Inc., Elmhurst, Ill.).

Labeling platelets with [^{3}H]5-HT or with [^{14}C]5-HT in vivo. This was performed either by the injection of 20 μCi of [^{3}H]5-HT (Amersham/Searle) into rabbits, or by bleeding donor rabbits and labeling suspension of washed platelets with [^{14}C]5-HT (New England Nuclear, Boston, Mass., specific activity 15.3 mCi/mmol). This compound was dissolved in distilled water and added to the first washing fluid (10 μCi/4×10^{10} platelets). The uptake of radioactivity amounted to 80% approximately after 5 min incubation at room temperature. The suspension of labeled platelets (7.5×10^9/rabbit) was injected into the rabbits 16 h before the experiment. On the other hand, rabbits whose platelets were labeled after serotonin infusion in vivo were subjected to experiments within 10–30 min after injection.

Measurements of serotonin radioactivity and of fibrinogen in circulating platelets of rabbit. Blood was obtained from the ear vein and mixed with ACD solution. Platelets were washed and adjusted to the final count of 10^9/ml. After sonication of platelet samples, 0.1-ml portions of the supernate were transferred to the vials and the radioactivity was counted in the liquid scintillation counter. The remaining volume of sonicated platelets was used for determination of platelet fibrinogen by the staphylococcal clumping method. Control experiments demonstrated that less than 1% of radioactivity was lost in washing fluids during isolation of platelets labeled with [5-^{3}H]HT in vivo.

Labeling platelets with [^{3}H]diisopropylfluorophosphate ([^{3}H]DFP) and study of the effect of SK on [^{3}H]DFP label in platelets. Blood was collected from three donor rabbits into acid citrate-dextrose solution (ACD). Suspension of washed platelets was adjusted to the final count of 3×10^9/ml. [^{3}H]DFP supplied by Amersham/Searle had specific activity of 3.3 Ci/mmol. 5 μCi of this compound was added to 12.5 ml of platelet suspension in the first washing fluid, which was subsequently divided into five equal portions. Then each of five receiver rabbits was injected with 2.5 ml of the labeled platelet suspension. After 16 h, and immediately before SK injection, 10 ml of blood was obtained from each rabbit and mixed with ACD. Another sample of blood was obtained 10 min after SK injection. Platelets were prepared from these samples of blood and adjusted to the final count of 10^9/ml. After sonication, radioactivity was counted in 0.1-ml samples.

The bleeding time in rabbits. The effect of SK on the bleeding time was studied in a mesentery preparation (12, 13). Rabbits were anesthetized with sodium pentobarbital (Diabutal, Diamont, Chicago, Ill.). After opening the abdominal cavity of rabbits, mesentery of the small intestine was pinned on the top of the microscope table (Carl Zeiss, Inc., New York.) The table was preheated to 37°C and the mesentery was washed with the Locke-Ringer's solution at the same temperature. The proximal mesenteric venule was transected with a sharp razor blade. The bleeding was observed with the help of microscopy (low magnification). The primary bleeding time was measured until its initial arrest. In every case, the transected vessels were observed for 30 min.

Statistical calculations. Means and standard deviations (SD, the square root of variance) were calculated by standard methods. Significance of differences between two means was calculated by Student's *t* test, those among several means by Duncan's multiple range test (14).

RESULTS

The effect of plasmin on platelets in vitro. The addition of plasmin to the washed platelets of rabbit produced their aggregation response, which reached its maximum after approximately 5 min. After this period of time, platelet clumps started to deaggregate (Fig. 2). The deaggregation was observed at all concentrations of plasmin studied. Incubation of platelets with plasmin resulted in the release of radioactivity from platelets labeled with [^{3}H]serotonin and in the release of adenine nucleotides (Table I). The bulk of the material was released during the first 3 min after addition of plasmin. On the other hand, LDH was retained in platelets during their incubation with plasmin. Because the level of LDH in plate-

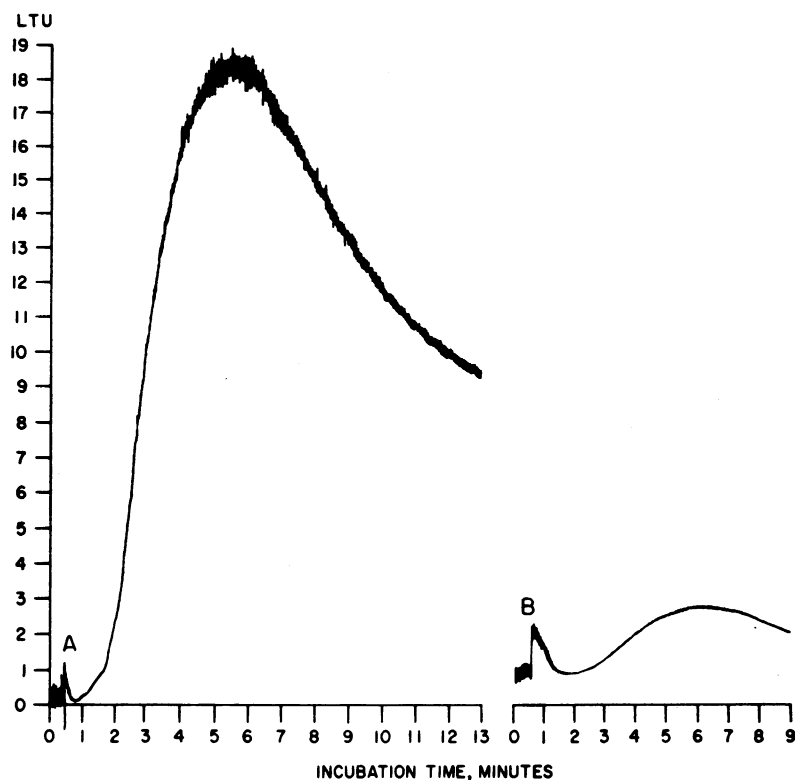

FIGURE 2 Aggregation and deaggregation of platelet suspension (10^9/ml) with plasmin. 0.1 ml of plasmin was added to 1.0 ml of platelet suspension (10^9/ml) as indicated by (A), 0.5% plasmin and (B), 0.1% plasmin (initial concentrations).

let sediment remained constant during the incubation of both intact and sonicated platelets with plasmin, it can be concluded that plasmin did not destroy platelet LDH. Also, plasmin did not release the radioactivity from platelets labeled with [^{3}H]DFP, but it caused their extensive aggregation.

In a few experiments plasmin was added to the suspension of rabbit platelets containing 0.02 M lysine. This was done to inhibit formation of plasmin precipitate which might affect platelet aggregation (see Methods). The rates of platelet aggregation and release of [^{3}H]5-HT radioactivity from platelets were not influenced by the presence of lysine in the platelet-suspending medium.

Table II shows that SBTI at the final concentration

TABLE I

*Effect of Pig Plasmin on the Release of Platelet Constituents**

Incubation time	Percent of total in the supernate							
	[5-^{3}H]HT radioactivity		Adenine nucleotides		LDH		[^{3}H]-DFP radioactivity	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
min								
0	1.5	0.5	2.5	2.5	0.57	0.48	4.8	2.9
3	40.6	13.6	31.3	2.1	0.53	0.36	4.5	2.6
15	53.7	15.6	50.8	15.6	0.68	0.42	5.9	4.1
No. of exps.	8		5		5		3	

* 1.0 ml of rabbit platelet suspension and 0.1 ml of 0.5% plasmin were incubated in aggregometer cuvettes with constant stirring at 37°C, and the released constituents were measured in supernatants obtained after centrifugation.

Table II

*Inhibitors of Plasmin-Induced Platelet Aggregation and Platelet Release Reaction**

	Final concentration	No. of exps.	Inhibition of the platelet aggregation‡		Inhibition of the release of [^{3}H]5-HT radioactivity§	
			Mean	SD	Mean	SD
			%		%	
SBTI	0.05%	9	99.6	±1.3	95.2	±9.9
EACA	10^{-1} M	4	85.4	±23.2	86.7	±20.9
EACA	10^{-2} M	5	76.5	±20.9	79.0	±26.5
Persantin	7×10^{-7} M	6	99.6	±0.9	99.3	±1.4
PGE_1	10^{-7} M	5	98.0	±1.5	99.0	±1.7
Phenylbutazone	3×10^{-3} M	7	85.4	±22.7	85.6	±15.9

* 1 ml of rabbit platelet suspension (10^9) was preincubated for 3 min with 0.1 ml of the solution of inhibitor or with 0.1 ml of Tyrode solution. Then 0.1 ml 0.5% pig plasmin was added and the mixture was incubated for another 3 min in a Payton aggregometer.

‡ Calculated by comparison of the light transmission changes in the control sample (plasmin + Tyrode) in the sample containing tested inhibitor.

§ Calculated by comparison of the release of [^{3}H]5-HT radioactivity in control sample (plasmin + Tyrode) and in the sample containing a tested inhibitor.

of 0.05% completely inhibited plasmin-induced platelet aggregation and platelet release reaction. EACA at the concentrations of 0.1–0.01 M and phenylbutazone (3×10^{-3}M) produced inhibition of the plasmin action on platelets. PGE_1 (10^{-7}M) and Persantin (7×10^{-7}M) appeared to be very potent inhibitors.

Table III shows that plasmin acting on either intact or sonicated platelets degraded approximately 90% of fibrinogen in 5 min. The addition of PGE_1 or Persantin to intact platelets resulted in the protection of at least 50% of the total fibrinogen. On the other hand, the addition of PGE_1 or Persantin to sonicated platelets did not inhibit the degradation of platelet fibrinogen by plasmin.

Table III

*Degradation of Platelet Fibrinogen by Plasmin and the Protective Effect of PGE_1 and Persantin**

Platelet pretreatment	Reagents added to platelets	No. of exps.	Platelet fibrinogen µg/10^9 platelets	
			Mean	SD
None	Tyrode	5	95.3	12.4
	Plasmin	5	7.7	4.4
	PGE_1 + plasmin	5	58.9	22.6
	Persantin + plasmin	2	59.5	—
Sonication	Tyrode	5	95.3	12.4
	Plasmin	5	7.5	6.5
	PGE_1 + plasmin	2	3.3	—
	Persantin + plasmin	2	3.9	—

* 1-ml samples of intact or of sonicated rabbit platelets were incubated with 0.1 ml of 0.5% plasmin for 5 min at 37°C in aggregometer cuvettes. The reaction was stopped with 0.1 ml 0.5% SBTI. PGE_1 or Persantin was preincubated with platelets for 1 min before plasmin addition. The final concentration of PGE_1 was 10^{-5} M, the final concentration of Persatin, 7×10^{-6} M.

Fig. 3 shows that the short preincubation of platelet suspension with plasmin enhanced the extent of ADP-induced aggregation. However, platelets incubated for a longer period of time with plasmin lost their sensitivity to ADP. It is also of interest to notice that after the platelets had been treated with plasmin, the change in shape resulting from the addition of ADP no longer occurred.

Platelets preincubated with plasmin became much less sensitive to thrombin and collagen. Using different concentrations of plasmin and different incubation times, we were unable to demonstrate a phase of increased sen-

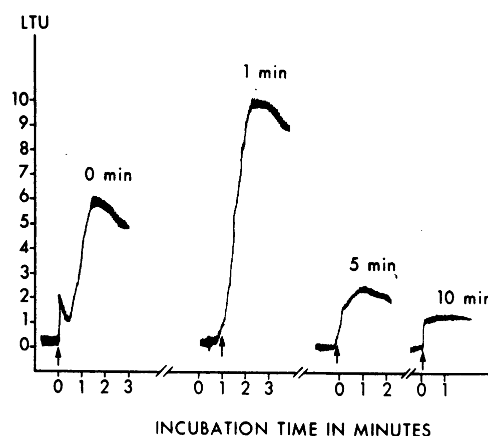

Figure 3 Effect of the preincubation of rabbit platelets with plasmin on ADP-induced aggregation. 1.0-ml samples of platelets (10^9/ml) were preincubated with 0.1 ml of 0.25% plasmin at 37°C (without stirring) for 1 min, 5 min, and 10 min. Light transmission was recorded automatically after addition of 0.1 ml ADP (10 µmol) to each sample.

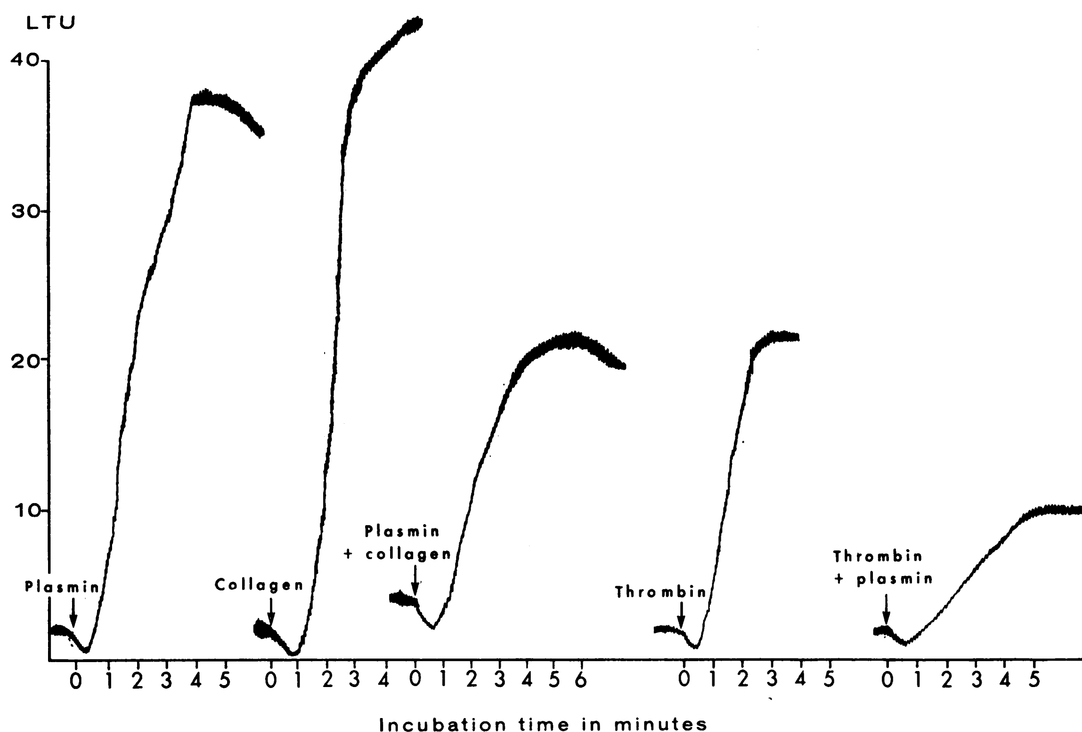

FIGURE 4 Light transmission changes in the suspension of rabbit platelets aggregated by plasmin, collagen, and thrombin. Following reagents were added to 1 ml samples of platelets: 0.1 ml of 0.5% plasmin; 0.1 ml collagen; 0.1 ml plasmin and 0.1 ml of collagen; 0.1 ml of thrombin (0.5 U/ml); 0.1 ml of thrombin (0.5 U/ml) and 0.1 ml of 0.5% plasmin.

sitivity of plasmin-treated platelets to thrombin and collagen. Fig. 4 shows the results of an experiment in which each of three stimuli, plasmin, collagen, and thrombin added to platelet suspension alone produced more aggregation than the mixture of collagen and plasmin or the mixture of thrombin and plasmin.

In the next experiment, pig plasmin was incubated with rabbit, pig, or human platelets for 3 min. The extent of platelet aggregation and the release of radioactivity from [^{3}H]5-HT-labeled platelets were similar in all three species (Table IV). Human plasmin activated by SK and pig plasmin had a similar effect on pig and human platelets. On the other hand, rabbit platelets were more resistant to the action of SK-activated plasmin. SK and plasminogen alone did not cause platelet aggregation and release of radioactivity even during incubation up to 7 min. Human plasminogen, contaminated with plasmin, produced only a slight change of platelet shape, as indicated by the amplitude of oscillations in the aggregometer. Aggregation of the suspension of washed human platelets by plasmin was also reversible. The level of platelet fibrinogen, as estimated by staphylococcal clumping method, was of the same order of magnitude in platelets of three species.

The effects of SK infusion to rabbits on platelets in vivo. The infusion of SK to rabbits in a dose of 10,000 U/kg body weight produced a pronounced activation of fibrinolysis in whole blood and in euglobulin fraction of plasma, and slight but significant prolongation of the thrombin clotting time of platelet-poor plasma (Table V). This dose of SK caused a significant drop of platelet count in whole blood between the 2nd and 5th min after injection (Table VI). The platelet count decreased to 75% of its initial value on the average. After 10 min the number of circulating platelets rose to the preinjection values. The injection of Tyrode solution in control rabbits did not produce any changes in platelet count.

In the following experiment rabbit platelets were labeled in vivo with [^{3}H]5-HT. The radioactivity (the number of counts per minute and per 10^{8} platelets) of the sample of washed platelet suspension prepared from blood obtained immediately before SK injection was taken as 100%. Table VII shows that the injection of sterile Tyrode solution to rabibts produced no significant drop of the ^{3}H radioactivity which amounted on the average to 24% after 10 min. Similar results were obtained in rabbits which had received infusion of platelets labeled with [^{14}C]5-HT in vitro, 16 h before SK injection.

In further experiments, rabbits which had their platelets labeled with [^{3}H]5-HT in vivo were injected either with Persantin in a dose of 7 mg/kg body weight or

TABLE IV

*Platelet Aggregation and Platelet Release Reaction Induced by Human SK-Activated Plasmin or by Pig Plasmin in Three Different Species**

Species of platelets	Enzyme added	No. of exps.	Aggregation (LTU)		[^{3}H]5-HT radioactivity release	
			Mean	SD	Mean	SD
					% in the supernate	
Rabbit	Plasminogen	5	0	—	1.04	0.62
	SK	4	0	—	1.9	0.9
	Plasminogen + SK	4	0.2	0.2	3.6	2.2
	Pig plasmin	5	16.6	13.6	38.6	15.4
Pig	Plasminogen	5	0.8	0.6	2.2	2.2
	SK	4	0	—	1.0	1.3
	Plasminogen + SK	4	23.0	19.0	43.5	25.4
	Pig plasmin	5	31.0	15.8	45.9	14.8
Human	Plasminogen	5	0	—	1.5	2.3
	SK	4	0	—	0.4	0.4
	Plasminogen + SK	4	19.6	10.4	47.2	37.9
	Pig plasmin	5	11.0	10.8	33.1	20.9

* 1-ml samples of platelet suspension (10^9) were incubated for 3 min with (*a*) 0.1 ml 0.25% plasminogen and 0.1 ml 0.9% NaCl: (*b*) 0.1 ml SK (500 *U*/ml) and 0.1 ml 0.9% NaCl: (*c*) 0.1 ml 0.25% plasminogen and 0.1 ml SK (500 U/ml); (*d*) 0.1 ml 0.5% pig plasmin. The mixture of SK and plasminogen was preincubated for 3 min before addition to the platelet suspension. In one experiment, incubation of rabbit platelet suspension for 7 min with 0.2 ml of 0.5% plasminogen activated with 500 U SK resulted in an aggregation of 7 LTU and in a release of 25.6% of [^{3}H]5-HT radioactivity.

with EACA in a dose of 500 mg/kg body weight. A control group of rabbits received no injection. Table VIII shows that the pretreatment of rabbits with either Persantin or EACA protected platelets against loss of ^{3}H radioactivity brought about by SK in the control group of rabbits. Pretreatment with Persantin did not inhibit the SK activation of euglobulin fibrinolysis and the whole-blood clot lysis. On the other hand, preinjection with EACA prevented the activation of fibrinolysis in clots made from the whole blood.

A group of rabbits received infusion of [^{3}H]DFP-labeled platelets. 16 h later these rabbits received standard injection of SK. Blood samples were obtained from these rabbits before and 10 min after SK injection to prepare platelet suspension and to count radioactivity (see Methods). Data presented in Table IX indicate no significant decrease of [^{3}H]DFP radioactivity in platelet suspension during a 10-min period after SK injection.

TABLE V

The Effect of SK Injection into Rabbits on the Fibrinolysis and Thrombin Time (Mean Values from 24 Rabbits and SD)

Test	Before SK		10 min after SK	
	Mean	SD	Mean	SD
Euglobulin fibrinolysis, *min*	>360	—	32.1	2.2*
Whole blood clot lysis, *min*	>360	—	4–30‡	—
Thrombin time, *sec*	11.4	2.8	16.3	7.4*

* Significant at the level of $P < 0.001$.
‡ Ranges of variations.

TABLE VI

Whole Blood Platelet Count in Rabbits after Injection of SK or Tyrode Solution

Material injected	Time after injection	Exp. no.	Platelet × 1000/mm^3	
			Mean	SD
	min			
Streptokinase (18 rabbits)	0	*1*	429.4	97.6
	1	*2*	392.2	43.2
	2	*3*	323.3	114.6
	5	*4*	336.7	130.5
	10	*5*	454.4	131.5
Tyrode (6 rabbits)	0	*6*	305.0	38.3
	2	*7*	308.0	33.1
	5	*8*	318.3	47.9
	10	*9*	301.7	47.0

P for *1* vs *3* <0.001; *1* vs. *4* <0.001.

TABLE VII

Loss of Radioactivity from Circulating Platelets Labeled with Serotonin after Injection of SK (10,000 U/kg Body Weight)

Solution injected	Exp. no.	Time after injection	Radioactivity in 10^8 platelets		Radioactivity in 10^8 platelets	
			Mean	SD	Mean	SD
			cpm		*% of initial radioactivity*	
Tyrode*		0	2,370	±866	100.0	—
	1	1	2,461	±956	103.4	±13.6
	2	10	2,326	±848	99.4	±13.4
SK*		0	2,412	±480	100.0	—
	3	1	2,100	±445	86.7	±10.7‖
	4	10	1,848	±452§	76.0	±8.3¶
SK‡		0	3,403	±517	100.0	—
	5	10	2,813	±559	82.5	±8.5

* Platelets labeled with [^{3}H]5-HT in vivo (mean values from nine experiments).
‡ Platelets labeled with [^{14}C]5-HT (mean values from five experiments).
§ The analysis of variance of the actual values of radioactivity using the Randomized Block Model provided a F statistic of 26.9 with 2 and 16 degrees of freedom. The probability of observing an F value at least as large as this is less than 0.001.
‖ P for *1* vs. *3* <0.05.
¶ P for *2* vs. *4* <0.005.

The experimental data presented in Table X show that the level of platelet fibrinogen in two control groups of rabbits amounted to 92.2 μg/10^9 platelets or 106.4 μg/10^9 platelets respectively. When Persantin was infused before SK injection the platelet fibrinogen dropped to 40.9 μg/10^9 platelets, whereas the control infusion of Tyrode's solution before SK injection resulted in a platelet fibrinogen of 14.5 μg/10^9 platelets. At the same time, the plasma fibrinogen by the staphylococcal clumping method did drop from 3.14 mg/ml to 1.80 mg/ml in a group of rabbits receiving Tyrode and from 3.11 mg/ml to 2.08 mg/ml in the Persantin-treated rabbits. The level of plasma fibrinogen determined as clottable protein was not changed. It can be seen that Persantin protected rabbits from losing an important portion of platelet fibrinogen but it did not affect the level of plasma fibrinogen.

In the following experiments, rabbit blood was collected and mixed with 3.8% sodium citrate in a proportion of 9 vol of blood to 1 vol of citrate. Subsequently, platelet-rich plasma was prepared from this blood to

TABLE VIII

*Loss of Radioactivity from Circulating Platelets Labeled with Serotonin after Injection of SK (10,000 U/kg body Weight) and the Protective Effect of Persantin and EACA**

Solution infused 10 min before SK injection	Time after SK injection	Radioactivity in 10^8 platelets		Radioactivity in 10^8 platelets	
		Mean	SD	Mean	SD
	min	*cpm*		*% of initial radioactivity*	
None*	0	2,412	±480	100.0	—
	10	1,848	±452	76.0	±8.3
Persantin, 7 mg/kg‡	0	3,450	±618	100.0	—
	10	3,568	±761	103.2§	±6.4
EACA, 500 mg/kg‡	0	3,500	±601	100.0	—
	10	3,425	±615	97.8§	±3.4

* Mean values from nine experiments.
‡ Mean values from six experiments.
§ Significantly different from the means of Exp. 1, $P < 0.005$.

Table IX

*Effect of the Injection of SK (10,000 U/kg Body Weight) to Five Rabbits on the Radioactivity of [^{3}H]DFP-Labeled Platelets**

	Time after injection			
	0 min		10 min	
	Mean	SD	Mean	SD
[^{3}H]DFP radioactivity, *cpm in 10^8 platelets*	294.2	±166.9	300.9	±159.1
[^{3}H]DFP radioactivity in 10^8 platelets, *% of initial*	100.0	—	103.3	±8.6

* Mean values from five experiments.

study platelet aggregation by collagen. Fig. 5 shows that the injection of SK caused a significant drop of platelet sensitivity to collagen, 10 min after injection. Infusion of Persantin (7 mg/kg weight) resulted in only a slight inhibition of collagen-induced aggregation. However, platelets obtained from rabbits receiving injection of both Persantin and SK were much more sensitive to collagen than the platelets from rabbits injected with SK alone.

The bleeding time from the proximal mesenteric venule of rabbits 10 min after SK injection was 5 times longer than that of the control rabbits injected with Tyrode solution (Table XI). Injection of Persantin in a dose of 7 mg/kg body weight and injection of EACA in a dose of 500 mg/kg body weight had no effect on the bleeding time. However, if Persantin was injected 10 min before SK injection, no significant prolongation of the bleeding time over controls was observed. Pretreatment of rabbits with EACA also exerted a protective effect which was, however, less pronounced.

DISCUSSION

It can be concluded that plasmin causes reversible platelet aggregation and release reaction as demonstrated by the release of [^{3}H]5-HT radioactivity and of adenine nucleotides from washed platelets. The LDH, a cytoplasmic marker, is retained in platelets, thus indicating that no lysis of platelets occurred. Plasmin action on platelets is inhibited as well by the inhibitors of plasmin (SBTI, EACA) as by the inhibitors of platelet release reaction (phenylbutazone, Persantin, PGE_1). In contrast, thrombin effects on platelets are not inhibited by SBTI and EACA but they are completely inhibited by hirudin (3). A short preincubation of platelets with plasmin enhances ADP-induced aggregation and in this respect plasmin acts similarly to thrombin (15, 16).

The loss of platelet sensitivity to ADP during longer incubation with plasmin may depend on the accumulation of adenine nucleotides in the suspending fluid and on the digestion of platelet fibrinogen.

Platelet fibrinogen undergoes almost complete degradation during plasmin action on platelets. The measurement of fibrinogen level in platelets by the staphylococcal clumping method gave similar values to those obtained by Keenan and Solum (17), who used immunoassay. Neither PGE_1 nor Persantin inhibits the plasmin action on purified fibrinogen or on the fibrinogen in platelet suspension homogenized by sonification. On the other hand, these drugs added to intact platelets protect at least 50% of platelet fibrinogen from the plasmin degradation. It can be suggested that PGE_1 and Persantin may protect platelet fibrinogen by inhibiting its release from platelets. It is likely that platelet fibrinogen is first released and then digested by plasmin. In the presence of PGE_1 or Persantin, plasmin may degrade only the fibrinogen adsorbed on the platelet membranes while fibrin-

Table X

Effect of the Infusion of SK (10,000 U/kg) on the Level of Platelet and Plasma Fibrinogen and the Protective Effect of Persantin

Solution infused 10 min before SK injection	Exp. no.	Time after SK injection	Platelet fibrinogen		Plasma fibrinogen: Staphylococcal clumping method		Plasma fibrinogen: Fibrin clot method	
			Mean	SD	Mean	SD	Mean	SD
		min	*µg/10^9 platelets*		*mg/ml*			
Tyrode	*1*	0	92.2	4.6	3.14	0.37	2.85	0.61
	2	10	14.5	2.5	1.80	0.83	3.01	0.53
Persantin, 7 mg/kg	*3*	0	106.4	9.0	3.11	0.31	2.95	0.44
	4	10	40.9	4.0	2.08	0.55	2.72	1.10

Platelet fibrinogen P for *1* vs. *2* <0.001; *3* vs. *4* <0.001 *2* vs. *4* <0.001.
Plasma fibrinogen (staphylococcal clumping method) P for *1* vs. *2* <0.02 P for *3* vs. *4* <0.005.
* Mean values from six rabbits.

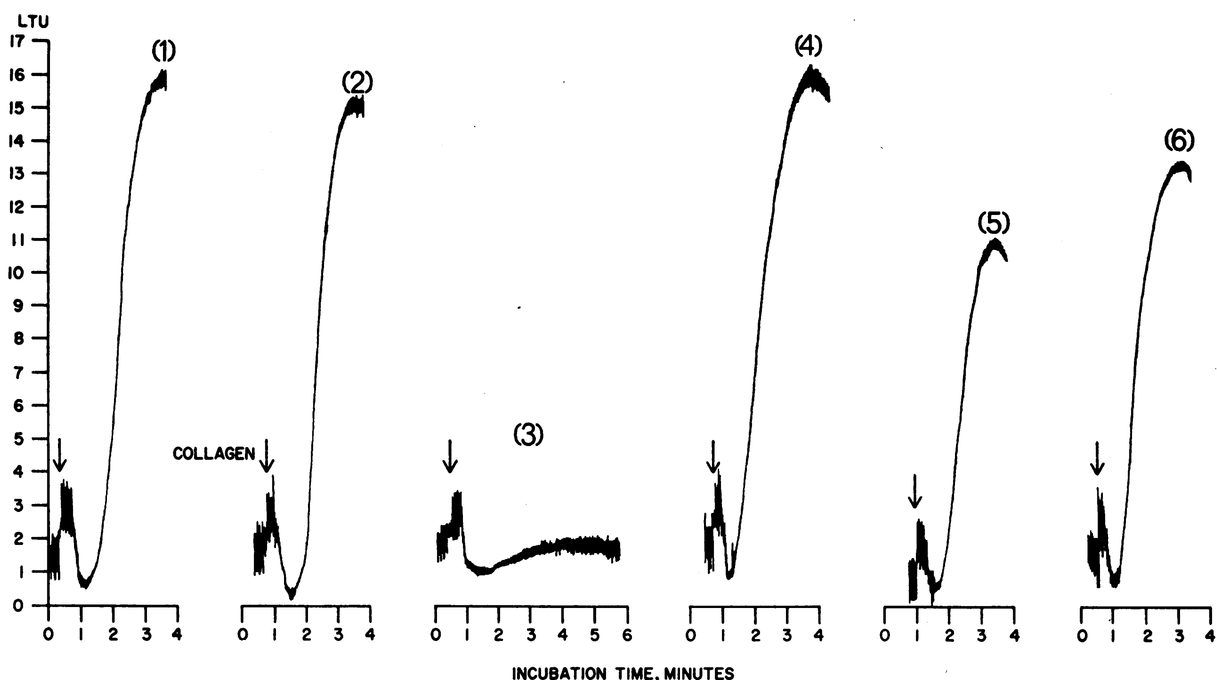

FIGURE 5 Decrease of the sensitivity of rabbit platelets to collagen after SK infusion, and the protective effect of Persantin. The addition of 0.1 ml of acid-soluble collagen to 1.0 ml of citrate, platelet-rich plasma is indicated by the arrows. Platelet count was adjusted to 5×10^8/ml. (*1*) Rabbit A, platelet aggregation in the preinjection sample. (*2*) Rabbit A, platelet aggregation 10 min after Tyrode injection. (*3*) Rabbit A, platelet aggregation 10 min after SK injection. (*4*) Rabbit B, platelet aggregation in the preinjection sample. (*5*) Rabbit B, platelet aggregation 10 min after Persantin injection. (*6*) Rabbit B, platelet aggregation 10 min after SK injection. Persantin or Tyrode was injected 10 min before SK. The dose of Persantin was 7 mg/kg weight, the dose of SK was 10,000 U/kg weight. This experiment is a representative one of six similar experiments. The differences between the extents of aggregation were statistically significant: P for *2* vs. $3 < 0.001$; P for *3* vs. $6 < 0.02$.

ogen located in the platelet granules is not accessible to the plasmin action. These findings are compatible with other reports that fibrinogen is adsorbed on the platelet membranes (4), and it is also a constituent of platelet granules (18, 19).

The difference between thrombin- and plasmin-induced platelet aggregates is that the latter deaggregate spontaneously. Plasmin also makes washed platelets less sensitive to thrombin and collagen. The relationship of these effects to the degradation of platelet fibrinogen or other platelet proteins by plasmin remains to be established.

Human, rabbit, and pig platelets are equally sensitive to pig plasmin; however, rabbit platelets are more resistant to human, SK-activated plasmin. The species specifity of plasmins may account for this difference. The activity of platelet antiplasmin is lower in rabbits than in pigs (20), and the level of platelet fibrinogen is of the same order of magnitude in three species.

Injection of SK into the rabbits activated plasminogen to plasmin, as indicated by the short euglobulin lysis time and lysis of clots formed from the whole blood. It appeared that this activation was not sufficient to destroy plasma fibrinogen since the level of thrombin-clottable protein was not changed after SK injection. On the other hand, thrombin time was slightly prolonged and the level of fibrinogen detectable by staphylococcal clumping test decreased. This could be explained assuming limited proteolysis of the fibrinogen molecule leading to the formation of fragment X and other early fibrinogen degradation products which are still clottable. However, fragment X clots more slowly with thrombin than fibrinogen does (21) and it is less reactive with staphylococcal suspension (22, 23).

Injection of SK caused a slight but significant drop of platelet count which occurred between the 2nd and 5th min after injection. Although it would be difficult to place any hemostatic significance on this numerical difference, it probably reflected transitory platelet aggregation occurring in the microcirculation. After 10 min, platelet count returned to normal values. It is likely that all platelet aggregates were disrupted at that time and that

all platelets were back in the circulation. This statement is supported by the same level of [^{3}H]DFP radioactivity in platelets before SK and 10 min after its injection. If the reversibility of plasmin-induced platelet aggregation occurs in vivo as well as in vitro, this phenomena may explain conflicting data in the literature regarding platelet counts after SK infusion (13, 24–26).

A single infusion of SK to rabbits caused a significant (about 25%) decrease of radioactivity in [^{3}H]5-HT or [^{14}C]5-HT labeled platelets in vivo. The platelet fibrinogen decreased by 85% after SK infusion and it is obvious that the decrease of platelet fibrinogen was much more pronounced than that of plasma fibrinogen. Pretreatment of rabbits with Persantin or with EACA inhibited the effect of SK on the release of platelet [^{3}H]5-HT radioactivity. Pretreatment with Persantin resulted also in a partial protection of platelet fibrinogen in rabbits injected with SK. These experiments suggest that plasmin formed in rabbit blood in vivo may act directly on the platelets and cause release of platelet constituents in vivo. As the result of the plasmin action on platelets in vivo, they become less sensitive to collagen. This could be counteracted by preinfusion of Persantin under the experimental conditions described in this study.

The question arises whether the action of plasmin on platelets in vivo may affect their hemostatic function. Rabbits infused with SK showed significant prolongation of the primary bleeding time from mesenteric vein and this observation was consistent with the findings by Hirsh, Buchanan, Glynn, and Mustard (13). These authors suggested that the digestion of fibrin by activated fibrinolytic enzymes in the hemostatic plug may be responsible for bleeding. However, Kjaerheim and Hovig (27) failed to demonstrate fibrin in the interior of hemostatic plug formed in the mesenteric vessels and they suggested that fibrin possibly exerts its action at a later stage of hemostasis, in sealing and enclosing the relatively vulnerable plug.

It is possible that the digestion of fibrin in the hemostatic plug is responsible for prolongation of the secondary bleeding. However, prolongation of the primary bleeding likely reflects platelet defects caused by plasmin. The nature of the plasmin-induced platelet defect, consisting of the decreased platelet sensitivity to collagen and the prolonged primary bleeding time, is not clear. The decrease of plasma and platelet fibrinogen could not account for these abnormalities since the concentration of fibrinogen necessary to support platelet aggregation appears to be much lower (28, 29). There is an intriguing possibility that after injection of SK, platelets have been rendered defective by a depletion of their storage pool. This possibility is supported by experiments in which Persantin was found to be a potent inhibitor of plasmin-induced platelet aggregation. Injection of Persantin protected rabbit platelets against the effect of SK. These rabbit platelets retained their serotonin and responded better to collagen. In addition, the SK-induced prolongation of bleeding time did not occur in rabbits receiving Persantin. Cucuianu, Nishizawa, and Mustard (30) found that higher doses of Persantin completely inhibited collagen-induced platelet aggregation and increased bleeding time. However, the dose used in our experiments (7 mg/kg) did not increase bleeding and produced only small inhibition of collagen-induced aggregation.

TABLE XI

*The Bleeding Time from the Proximal Mesenteric Veinule of Rabbits Infused with SK and the Protective Effect of Persantin and EACA**

Solution infused to rabbits	Exp. no.	Bleeding time Mean	Bleeding time SD
		sec	
Tyrode	*1*	106.3	±55.6
SK, 10,000 U/kg	*2*	572.3‡	±204.9
EACA, 500 mg/kg	*3*	94.8	±85.6
Persantin, 7 mg/kg	*4*	115.0	±84.1
Persantin 7 mg/kg + SK, 10,000 U/kg	*5*	104.3§	±42.9
EACA, 7 mg/kg + SK 10,000 U/kg	*6*	253.3‖	±236.2

* Mean values from 12 rabbits in each group. In groups 5 and 6, persantin and EACA were injected 10 min before SK.
‡ P for *2* vs. *1* <0.001.
§ P for *5* vs. *2* <0.001.
‖ P for *6* vs. *2* <0.005.

It is generally accepted that the bleeding in patients receiving SK may depend on several causes, such as digestion of fibrinogen, digestion of fibrin in the hemostatic plug (13), and the formation of fibrinogen degradation products that may inhibit clotting (21, 31–33) and platelet aggregation (25, 33–37). However, recent observations indicate that the "antiaggregating activity" of degradation products is low and not specific (3, 38).

Our experiments suggest that the platelet defect resulting from the plasmin action may be an important cause of bleeding in patients receiving thrombolytic therapy as well as in patients with systemic activation of fibrinolysis due to other factors. It can also be suggested that agents inhibiting platelet release reaction, such as Persantin, could be used in certain circumstances for the control of bleeding. The dosage of persantin administered to rabbits was at least 10 times higher than the standard dosage of this drug used in human therapy (39). It is difficult to extrapolate from the study on animals to clinical conclusions. It seems, however, that our results warrant further investigations on the simultaneous use of SK and other antiplatelet agents in animals and in man.

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