A Streptokinase Cofactor in Human Platelets.* (27168)

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Tillett and Garner(1,2) demonstrated that certain hemolytic streptococci elaborate an extracellular substance, streptokinase or SK, which can rapidly liquefy human plasma clots. Milstone(3) demonstrated that SK was not itself fibrinolytic but required the presence of a "lytic factor" from human serum. These observations were expanded by Christensen(4) who considered that SK was a direct activator of human profibrinolysin or plasminogen. Many observers noted that SK readily produced fibrinolysin in plasma fractions from certain animal species (human, cat, dog, monkey, rabbit) but not in others (horse, pig, sheep, goat, guinea pig, cow). The next big step in understanding the fibrinolytic enzyme system was the suggestion by Mullertz and Lassen(5) that SK reacts with a proactivator, abundantly present in human plasma but absent in bovine plasma, and that the "activator" so formed can convert human or bovine profibrinolysin to fibrinolysin. In the last few years considerable controversy has developed as to whether human proactivator has a separate physical identity from human profibrinolysin or whether these two activities derive from the same molecule(6,7, 8,9). Much of the feeling that proactivator and profibrinolysin are identical derives from the inability to separate 2 distinct activities from human serum by current fractionation methods.

The experiments described herein show that well washed human platelets contain a factor, which, in the presence of small amounts of SK, is not itself fibrinolytic but is able to produce fibrinolysin from bovine serum euglobulin. In this communication, this property is called "SK cofactor". The term "activate profibrinolysin" has been avoided as our preliminary experiments do not demonstrate the mechanism involved.

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The method used for assay of fibrinolysin was designed to measure fibrinolysis in a system as free from contaminants as possible. Bovine fibrin was exhaustively washed and iodinated, the solubilization of I^{131} serving as a measure of fibrinolysin activity.

Materials and methods. Buffered saline: 9 parts of 0.85% sodium chloride and 1 part of imidazole buffer pH 7.4. *Streptokinase*: Varidase® supplied through the courtesy of Dr. Ruegsegger, Lederle Laboratories. *Human platelets and platelet extract*: Platelets were concentrated from EDTA anticoagulated blood by the usual differential centrifugation procedures, washed from 6 to 12 times in 0.85% saline and resuspended in a volume equal to one-fifth of that of the initial platelet-rich plasma. Platelet extract was prepared after rupturing the platelets by 10-minute agitation (Vortex Jr. Mixer) with fine glass beads and discarding the materials sedimented at 20,000 g for 30 minutes. *Euglobulin*: Oxalated bovine or human plasma was recalcified, the fibrin removed, and the serum treated by adsorption with barium sulfate. The euglobulin fraction was then obtained by diluting this serum with 19 volumes of distilled water, adjusting the pH to 5.3, chilling, collecting the precipitate and redissolving at pH 7.3 in one-tenth the original serum volume. This crude serum fraction was stored at –20°C., thawed and diluted with 9 volumes of buffered saline before use. *Continuous flow electrophoretic fractionation* employed previously described technics(10). *Fibrin plate method* was slightly modified from that of Astrup and co-workers(11) and employed Armour Bovine Fraction I as the fibrinogen. *Fibrin I^{131} method*: Fibrin obtained from recalcified oxalated bovine plasma was squeezed as free from serum as possible and dispensed in 0.85% saline in a Waring Blendor. The insoluble fibrin was recovered by centrifugation, then dispensed and rewashed at least 10 times after the wash solution appeared clear. The finely divided fibrin was dried by alter-

nate applications of alcohol and ether in a Büchner filter, ground in a mortar and stored at –20°C. Suitable quantities were iodinated by the tri-iodide method(12), and free I^{131} removed by dialysis and many washings.

For assay purpose fibrin I^{131} was so suspended in buffered saline that each 2 ml contained approximately 2 mg fibrin giving a CPM of 70,000 to 100,000. The fibrin was dispensed with an automatic pipette from a constantly agitated reservoir. Each assay tube was precounted to assure an equal distribution of the suspension and to allow a final calculation of percent of I^{131} solubilized (equivalent to percent fibrin lysed). Three ml of materials to be tested were added to the 2 ml fibrin I^{131} and the mixture incubated at 37°C in a glass constant temperature bath placed upon a large magnetic stirrer. Individual stirring in each tube was accomplished by inserting a flattened staple. After one hour incubation the tube contents were promptly filtered (No. 42 Whatman paper) and radioactivity measured in 1 ml samples of filtrate. Determinations were carried out with a Picker X-Ray automatic sample changer, well scintillation counter, and spectro scaler with automatic write-out. A single lot of iodinated fibrin proved suitable for use during a 2-week period. Although the fibrin concentration per test was increased as I^{131} decayed the exact concentration was not critical, the same percentage of fibrin being lysed by a standard enzyme preparation when the substrate tubes contained amounts varying from 1 to 5 mg.

Results. Demonstration of SK cofactor in human platelets: Table I shows the lytic effects of 6-times washed platelets and SK (50 u/ml) on plates containing plain fibrin (BF), fibrin heated at 85°C for 45 minutes (BF – 85°) and fibrin enforced with concentrated human euglobulin (HBF). SK alone lyses only HBF plates. SK plus platelets lyse either BF or HBF, but not BF – 85°C suggesting the necessity for the presence of a heat-labile lytic precursor.

TABLE I. Lysis of Fibrin Plates by Human Platelets and SK.

Mixture	Plates, lysed area (mm^2) BF	BF–85°C	HBF
Platelets* + SK (50 u/ml)	144	0	240
Buffer + "	0	0	240
Platelets + buffer	0	0	0

* Whole platelets, washed 6 ×, concentration = 2×10^6 per mm^3.

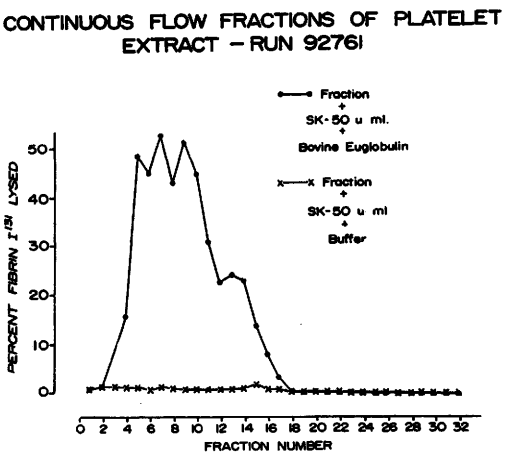

FIG. 1. SK cofactor activity in fractions of human platelet extract prepared by continuous flow electrophoresis (pH 8.6).

A similar experiment is shown in Table II. Here whole platelets or platelet extract were mixed with SK and/or bovine serum euglobulin before application to heated fibrin plates or testing for lysis of fibrin I^{131}. Platelet extract was less potent than whole platelet suspension.

Platelet fractions: Platelet extract was subjected to continuous flow electrophoresis and the resultant 32 fractions tested for SK-cofactor activity. Fig. 1 demonstrates the results in a typical experiment. None of the fractions contained lytic factor since no lysis occurred when they were mixed with SK *without* added bovine euglobulin. Certain fractions did contain SK cofactor since lysis of fibrin was observed in the presence of bovine euglobulin. The tubes in which this activity was found contain protein similar in mobility to serum γ and β globulins.

Stability of platelet cofactor: Platelet extracts heated to 56°C for 5 minutes lost all activity. Storage at –20°C usually preserved activity for up to 2 weeks.

Comment. Human platelets or platelet fractions and streptokinase are not fibrino-

TABLE II. Lysis of Heated Bovine Fibrin Plates and Fibrin I^{131} by Equal Mixtures of Whole Platelets or Platelet Extract, SK and Bovine Serum Euglobulin.

Contents of mixture:					
Whole platelets	Platelet extract	SK, 50 u/ml	Bovine euglobulin	BF-85°, plate area lysed, mm^2	Fibrin I^{131}, % lysed
+	0	+	+	36	45.6
+	0	+	0	0	.9
+	0	0	+	0	.4
+	0	0	0	0	.5
0	+	+	+	25	38.9
0	+	+	0	0	1.5
0	+	0	+	0	1.5
0	+	0	0	0	.4
0	0	+	+	0	1.0
0	0	+	0	0	.1
0	0	0	+	0	.6
0	0	0	0	0	0

lytic but when a bovine serum fraction is added a potent fibrinolysin appears. The experiments do not demonstrate the mechanism whereby this activity develops, hence terms such as proactivator and profibrinolysin are avoided.

These washed human platelet suspensions or extracts were essentially free of antifibrinolytic activity. Antifibrinolysin in bovine platelets has been demonstrated by Johnson and Schneider(13) and Alkjaersig(14). Stefanini and Murphy(15) found antifibrinolytic activity of human plasma proportional to the platelet content *but* no antifibrinolysin in washed human platelets. Hume(16) also presented evidence that human platelets might exert an inhibitory effect on fibrinolysis.

Tissue activators of the fibrinolytic enzyme system, described extensively by Astrup's group(17,6) and others (18,19) differ from platelet cofactor in mode of action, stability and solubility.

The presence of SK cofactor in platelets may prove of some therapeutic importance. Platelets are incorporated in propagating thrombi and during SK therapy might serve as foci for fibrinolysis. No *in vivo* studies have been made.

Conclusions. Human platelets together with streptokinase and bovine serum euglobulin produce a potent fibrinolysin. In the absence of any one of these 3, no fibrinolysis is observed.

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