The Plasma Plate Method for Estimating Thrombolytic Activity.* (27636)

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This report deals with a standard human plasma-clot thrombolytic method, based upon the principles used in the fibrin plate method of Astrup and Müllertz(1). It measures the ability of a given test material to produce measurable lysed areas in the thin clotted plasma layer.

Terminology. To the well-known terms in this field, we have recently(2) added the term 'plasminoplastins' for those 'activators' which convert plasminogen into plasmin *directly*, thus distinguishing them from 'lysokinases' which require *two steps*. 'Thrombolysis'(3) is now widely used to denote dissolution of a thrombus, rather than of isolated fibrin ('fibrinolysis').

Materials and methods. Outdated human plasma. A pool of one liter of at least 5 outdated (blood bank) plasmas was adjusted to pH 7.4, divided into 10 ml samples and kept frozen (−20°C) until used within 6 months. In this study 2 one-liter batches (labeled No. 1 and 2) of such plasma were used.

Borate buffer (modified) pH 7.6 was made by mixing in a volumetric flask 5g $Na_2B_4O_7 \cdot 10\ H_2O$, 11.25g H_3BO_3, 1.4g $CaCl_2$, 12g dextran, 2.25g NaCl and distilled water q.s. 1 liter.

Thrombin (bovine) was supplied through the courtesy of Dr. J. T. Correll, Upjohn Co., unitage according to manufacturer.

Petri dishes with 9 cm diameter were used. These were autoclaved and kept under sterile conditions at 4°C.

Streptokinase (SK) was Lederle's 'Varidase'. The dry material was dissolved in distilled water to give 10,000 units/ml.

Human plasminogen was prepared by Kline's(4) procedure from human plasma Fraction III and further purified by adjusting to pH 2, heating at 100°C for 30 minutes and precipitating with 1M NaCl. The specific activity of the purified plasminogen was about 80 casein units/mg N.

Human plasmin with specific activity of 75 casein units/mg N was prepared and further purified as previously described(2).

Human plasmin—NIH, was supplied through the courtesy of Dr. W. G. Workman and assayed 9.5 casein units/ml.

Urine plasminoplastin (UP) or urokinase was supplied through the courtesy of Dr. H. O. Singher, Ortho Laboratories.

Trypsin was a commercially available (Worthington's cryst. trypsin) twice crystallized, salt-free, after Kunitz and Northrop (1936).

Plasma samples were secured, using a silicone technic, from human venous blood collected in lusteroid tubes containing 1/10 volume 3.2% trisodium citrate, and tested immediately.

Fibrin plate method. The well known Astrup-Müllertz standard fibrin plate method (1) as modified by Alkjaersig *et al.*(5) was used. The fibrinogen was Armour's bovine Fraction I.

Plasma plate method. Two ml of plasma, thawed at room temperature, were mixed with 8 ml borate buffer in a petri dish and the mixture clotted with 0.1 ml thrombin (100 units/ml). The plate was covered, placed on an horizontal bench and allowed to form a firm clot. After about 20 minutes, using 0.2 ml pipette, three 30 lambda drops of test material were placed on the surface of the clot. One plate was needed for each test material. The plate was incubated for 20 hours at 34°C. The product of 2 perpendicular diameters of the lysed zone was recorded and the mean value of the 3 determinations was calculated. The plasma-clot contains the precursors of the fibrinolytic system as well as the inhibitors.

Results. A comparative study was made by testing trypsin, human plasmin-NIH, human plasmin, urine plasminoplastin and streptokinase by (a) the plasma plate and (b) the fibrin plate methods. The typical results shown in Table I indicate that the plasma

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TABLE I. Sensitivity of Plasma Plate Method to a Number of 'Thrombolytic Agents' Compared to Fibrin Plate Method.

Preparation tested	Lysed area in mm²	
	Plasma plate method	Fibrin plate method
Trypsin, 200 μg/ml	0*	779
20 "	0	208
2 "	0	0
Human plasmin–NIH, 8 u/ml	0	681
4 "	0	399
Human plasmin, 20 u/ml	76	1363
10 "	0	750
5 "	0	542
UP, 50 μg/ml	485	1468
25 "	49	894
10 "	0	576
SK, 5000 u/ml	892	0
1000 "	534	0
500 "	406	0
100 "	175	0
10 "	72	0

* Larger amounts of trypsin may give some plasma clot lysis.

plate method is especially sensitive to SK, less sensitive to UP, and not susceptible to the proteolytic enzymes in the concentrations used. Duplicate assays were run with SK 1000 units/ml and 100 units/ml, using as substrate (a) 12 different outdated plasmas and (b) batch No. 2 of pooled outdated plasma (Table II). The values obtained with the 12 different plasmas, although not significantly different statistically ($P>.05$) from those obtained with the batch No. 2 plasma, were divergent enough to indicate a preference for restricting quantitative tests to a single pool of plasma.

The lytic activity of plasma from 22 subjects following exercise (running 10 miles per hour on a treadmill for 6-32 minutes) was tested. The post-exercise plasma lysed human plasma-clots in 17 out of 22 experiments, but lysed bovine fibrin-clots in only 5 of these experiments (Table III). Differences between the two batches of plasma were insignificant ($P>.05$). The new methodology, therefore, is applicable to a study(6) of effects of exercise on the fibrinolytic system.

TABLE III. Plasma Thrombolytic Activity of Healthy Adults after Exercise Measured by the Plasma and the Fibrin Plate Method. Plasma batch No. 1 and No. 2 designate the batch of outdated plasma from which the plasma-clot was formed.

Plasma tested from subjects after exercise	Lysed area in mm²		
	Plasma plate method		Fibrin plate method
	Plasma Batch #1	Plasma Batch #2	
1.	91	90	0
2.	134	141	0
3.	0	0	0
4.	174	175	60
5.	124	124	0
6.	67	74	0
7.	189	194	155
8.	0	0	0
9.	0	0	0
10.	181	186	108
11.	129	141	42
12.	0	0	0
13.	88	86	0
14.	298	290	0
15.	94	85	0
16.	0	0	0
17.	312	321	158
18.	133	128	0
19.	175	165	0
20.	151	157	0
21.	167	178	0
22.	58	65	0

TABLE II. Plasma Plate Method. Effect of the plasma from which the clot was formed on thrombolytic activity of 2 concentrations of SK.

	Streptokinase, 1000 u/ml		Streptokinase, 100 u/ml	
	12 different plasmas*	Plasma* Batch No. 2	12 different plasmas*	Plasma* Batch No. 2
Range	465–861	420–598	163–414	151–235
Mean ± stand. dev.	630 ± 264	494 ± 116	263 ± 170	187 ± 45

* Plasma from which the substrate clot was formed.

SK produces plasminoplastin and plasmin in activating human plasminogen. In activating human plasma, however, little, if any, plasmin is demonstrable, although high plasminoplastin levels are attained. Taking these facts into consideration, the following experiment was performed. Normal human plasma and human plasminogen were activated with different amounts of SK and tested

TABLE IV. Thrombolytic and Fibrinolytic Activity as Measured by Plasma and Fibrin Plate Method of: (1) SK, (2) SK-Human Plasma and (3) SK-Human Plasminogen (H. Plgn).

Preparation tested	Lysed area in mm²	
	Plasma plate method	Fibrin plate method
SK, 1000 u/ml	507	0
SK, 100 "	155	0
SK, 10 "	64	0
SK, 1 "	0	0
Human plasma	0	0
SK, 1000 u/ml + plasma	525	1190
SK, 100 " "	117	415
SK, 10 " "	0	144
SK, 1 " "	0	50
H. Plgn, 4 cas. u/ml	0	0
Idem + SK, 1000 u/ml	547	1000
" + SK, 100 "	197	717
" + SK, 10 "	46	289
" + SK, 1 "	0	172

by the plasma and fibrin plate methods (Table IV). Despite the formation of (a) plasminoplastin in plasma, and (b) plasminoplastin and plasmin in plasminogen solutions (see fibrin plate method), the lytic areas obtained with the plasma plate method by SK-plasma and SK-plasminogen were not significantly larger than those obtained with SK alone.

Discussion. The foregoing data provide evidence that the plasma plate method is reproducible and specific for a certain category of 'thrombolytic' agents. In Table I, insensitivity to the stated amounts of trypsin and plasmin indicates inhibitors of the active enzymes, which retain their function although trapped in the plasma clot. Inactivity of small amounts of UP also suggests trapped natural inhibitor(s). The theory(7) that fibrin can reverse inhibition of plasmin by antiplasmin finds no support in the above data. Of the agents used in the present study, only SK was efficiently *thrombolytic* (referring to lysis of *plasma clots*). Table IV shows insignificant additional plasma clot lysis when SK was tested in the presence of *added* human plasma or human plasminogen, in amounts which gave very marked lysis in the fibrin plates.

The evidence (Table II) that the reactivity in the clot lysis test is sufficiently uniform, especially when using a single pool of outdated human plasmas, indicates that the method can be put to practical use (*e.g.*, Table III), without going into the technical problems such as relative proportions of inhibitors, possible effects of platelet residues, etc., which may be involved in determining "reactivity." Use of plasma-clots afforded a more sensitive indication of the alteration of the lytic system in plasma after stressful exercise than did fibrin clots. The differences will be investigated as to whether there are: (a) differences in available plasminogen levels, (b) preferential activation of human, compared with bovine, plasminogen, or (c) a two-step activation, in which the thrombolytic factor in plasma first acts on a precursor to form a 'plasminoplastin', which then converts plasminogen to plasmin. This last would be expected of a 'lysokinase' type activator(6).

Summary. A simple, reproducible and sensitive method for determining plasma thrombolytic activity by measurement of the lysed areas produced in a thin layer of human plasma-clot has been described. This method was insensitive to considerable concentrations of trypsin or plasmin, moderately sensitive to UP, but very sensitive to SK. In 22 tests of post-exercise plasmas, lysis occurred in 17 with the plasma plate method, compared with 5 using the bovine fibrin plate method.

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