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Research Article

STUDIES ON ENHANCED FIBRINOLYTIC ACTIVITY IN MAN *

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Over the years there have been reports describing the occurrence of increased fibrinolytic activity in the blood of both humans and experimental animals in association with a wide variety of circumstances which include wet cupping (1), peptone injections (2), operations (3), emotion (4), exercise (5), adrenaline injections (5) and sudden death (6, 7). In the past decade low levels of fibrinolytic activity have been observed to occur spontaneously in normal, nonstressed adults (8) while significantly increased levels of activity have been noted following acetylcholine injections (9), electroshock (10), circulatory collapse (11), pyrogen reactions (12), local ischemia (13, 14), pulmonary surgery (15) and such obstetrical complications as amniotic fluid embolism, *abruptio placentae* and fetal death *in utero* (16).

The reports mentioned in the preceding paragraph represent only a few of the circumstances in which increased fibrinolytic activity has been observed but illustrate the diverse nature of the clinical and experimental factors that have been reported to produce increased fibrinolytic activity. The mechanism underlying this activity has not been elucidated, but has been variously ascribed to either *1*) a reduction in the antiplasmin level of the blood or *2*) the presence in the blood of an activator of plasminogen.

Recent advances in the basic knowledge of the fibrinolytic enzyme system of human plasma (17, 18) and the development of new assay techniques have offered an opportunity to reinvestigate the occurrence and mechanism of enhanced fibrinolytic activity in patients following various stimuli.

A study was therefore carried out to determine the influence of electroshock, pyrogens, epinephrine, acetylcholine, ischemia and exercise upon the blood fibrinolytic system. It was shown that enhanced fibrinolytic activity occurred in the blood of almost all patients following electroshock, pyrogens and severe exercise and that a less consistent response occurred following epinephrine, acetylcholine and ischemia. Furthermore, it was possible to demonstrate the appearance in the plasma of a plasminogen activator following each of the circumstances mentioned above. Finally, it is suggested on the basis of the data that the enhanced fibrinolytic activity observed following these stimuli was not due to the presence of increased amounts of circulating plasmin but, rather, to the presence of an increased level of circulating activator.

MATERIALS AND METHODS

Biochemical studies

A variety of assay procedures was employed to test most samples. Unless otherwise designated, all blood specimens were collected from an antecubital vein, oxalated and immediately refrigerated in an ice chest until assayed.

I. Assays for fibrinolytic activity. a) Whole blood clot lysis. Whole blood clots were made with 0.9 ml. of oxalated blood and 0.1 ml. of thrombin (Parke-Davis; 10 units per ml. in 0.01 M veronal buffer, pH 7.4). Clots were incubated at 37° in a water bath and the time for complete lysis recorded.

Samples of spontaneously clotted whole blood were compared with oxalated samples clotted with thrombin. In most instances, the former were found to have longer lysis times [1] and for reasons of convenience the latter technique was utilized throughout these experiments.

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[1] When oxalated blood was clotted with thrombin in the presence of calcium, the lysis time was prolonged as compared to specimens without calcium. An explanation for this difference is suggested by the studies of Medart (19) who demonstrated that in the presence of calcium ions, fibrinogen and fibrin are made more resistant to the action of proteolytic enzymes. Since the submission of this manuscript, similar observations have been reported by Fearnley and Ferguson (Clin. Sci. 1958, 17, 555) and the effect attributed to the inhibitory action of calcium on fibrinolytic activity.

b) *Plasma euglobulin clot lysis.* The plasma euglobulins were precipitated from 0.5 ml. plasma according to the method of Milstone (19 volumes water containing 0.32 volume 1 per cent acetic acid added to 1 volume plasma) (*20*). After centrifugation, the precipitate was resuspended in 0.5 ml. 0.01 M veronal buffer, pH 7.4, and clotted with 0.5 ml. of thrombin (2 units per ml.). Clots were incubated at 37° C. and the time for complete lysis recorded.

This technique was found to be very sensitive for assaying the small amounts of fibrinolytic activity in plasma and was therefore extensively utilized in these studies. Since with fibrinolytic assays, the activity is a direct function of the reciprocal of the lysis time (17), a logarithmic plot of the lysis time *vs.* units of activity exhibits a linear relationship. Utilizing such a plot, and arbitrarily assigning an activity of 1 unit to a lysis time of 30 minutes, lysis times observed with the plasma euglobulin clot lysis technique were converted into units of fibrinolytic activity. All lysis times greater than 300 minutes are referred to as less than 0.1 unit.[2]

c) *Fearnley clot lysis technique* (*21*). Employing sterile pipettes and working in a cold-room as proscribed by Fearnley, Revill and Tweed (21), 0.5 ml. plasma samples were serially diluted from 1 : 4 to 1 : 32 in 0.1 M veronal buffer in saline, pH 7.4, and were then clotted with 0.5 ml. thrombin (2 units per ml.). Clots were incubated at 37° C. and the time of complete lysis recorded.

This method was found to be more cumbersome and less sensitive than the plasma euglobulin method and possessed the further disadvantage of employing several dilutions of plasma.

d) *Astrup fibrin plate* (*22*). As with previous techniques, this method, which involves the measurement of the area of lysis produced by test solutions placed upon a standard, unheated, bovine fibrin plate, does not distinguish between plasminogen activators and plasmin. It was found to be relatively insensitive for measuring the fibrinolytic activity in whole plasma since incomplete zones of lysis were produced. However the application of the *plasma euglobulin* from the plasma of patients with enhanced fibrinolytic activity frequently produced complete zones of lysis.

e) *I^{131}-Trace-labeled human plasma clots* (*23*). This method involves the measurement of the lysis of preformed human plasma clots, enriched with small quantities of purified human plasminogen and tagged with trace amounts of bovine I^{131}-labeled fibrinogen. The clots, formed upon wire-spirals, were incubated at 37° C. in the test solution (plasma, or euglobulin solution), after which the clots were removed, washed with 0.9 per cent saline, and the radioactivity released into the supernatant determined.

The lysis of these clots has been demonstrated to be directly related to the level of plasminogen activator in the test solution and to be relatively insensitive to the presence of plasmin (*23*).

II. Demonstration of plasminogen activator in plasma. Activators of plasminogen in plasma were demonstrated by studying the rate of activation of plasminogen in an activation mixture consisting of purified human plasminogen and the patient's plasma euglobulin solution. The human plasminogen was added to make available a uniform excess of substrate. The use of the euglobulin fraction, rather than whole plasma, as the source of activator, obviated the masking effects of plasma inhibitors. Plasminogen was prepared according to the method of Kline (24). Identical 0.2 ml. aliquots of a purified plasminogen preparation containing 9.70 casein units per ml. (25, 26) were incubated at room temperature with 1.8 ml. of resuspended plasma euglobulin in 0.01 M veronal in 0.9 per cent saline, pH 7.4. At intervals during the incubation period, 0.2 ml. aliquots were removed and added to tubes containing 0.7 ml. of 0.3 per cent bovine fibrinogen[3] in buffer, and clotted with 0.1 ml. of buffered thrombin (10 units per ml.). The lysis times of these clots, incubated at 37° C., were then determined and expressed in activity units (as defined for the plasma euglobulin clot lysis technique).

In samples containing an activator of human plasminogen, there was a progressive shortening of the clot lysis time.[4] Figure 1 illustrates the sensitivity of this technique to small amounts of purified urokinase,[5] a known plasminogen activator, and the lack of activation produced by relatively large amounts of spontaneously activated purified human plasmin (28) or in the absence of urokinase (control).

III. Assays for plasma plasminogen, plasmin, antiplasmin and fibrinogen. a) *Plasminogen.* Plasminogen was measured by conversion to plasmin with optimal amounts of streptokinase and assay of the plasmin formed. Two techniques were employed:

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[2] Most specimens from "normal" adults will lyse in from 6 to 24 hours by this technique; however, shorter or longer lysis times are not unusual.

[3] Kindly supplied to us by Dr. Kent Miller and containing 92 per cent clottable fibrinogen. The sensitivity of this technique is significantly affected by the purity of the fibrinogen preparation employed. A fibrinolytic assay was employed since the measurement of the small quantities of plasmin formed during the activation required a highly sensitive technique.

[4] In the presence of an activator, but not in its absence, activation of the plasminogen in the plasma euglobulin solution alone could be demonstrated, but the addition of purified human plasminogen, as above, markedly increased the sensisivity of the technique, *e.g.*, in one experiment the fibrinolytic activity increased 1.61 fibrinolytic units in a 30 minute period when the extra plasminogen was present, and only 0.42 unit in its absence. Although a progressive increase in fibrinolytic activity implies the formation of plasmin, similar findings could be obtained if increased amounts of activator were formed.

[5] Kindly supplied by Dr. J. Ploug, Leo Pharmaceutical Co., Copenhagen. The preparation contained 5,100 units per mg. (27).

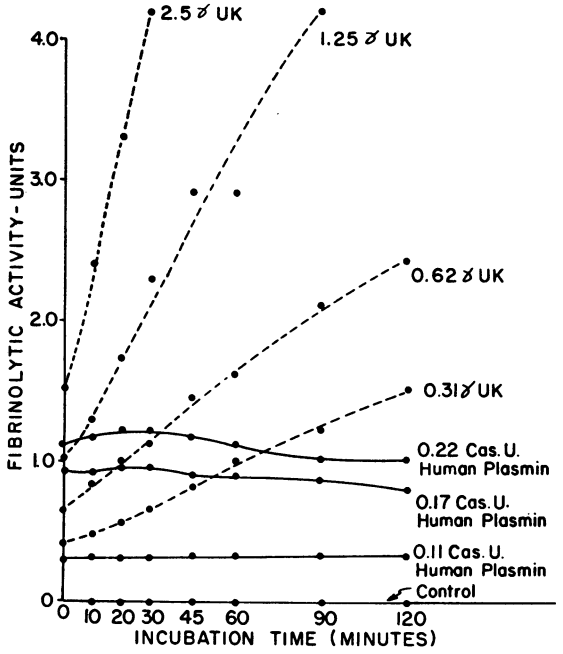

FIG. 1. ACTIVATION CURVES OBTAINED WITH HUMAN PLASMINOGEN PLUS UROKINASE

Varying amounts of urokinase incubated with purified human plasminogen at 25° C. Aliquots removed at time intervals and tested for fibrinolytic activity in a bovine fibrinogen-thrombin clot system. Fibrinolytic activity expressed in fibrinolytic units (see text). No activation occurs with human plasmin alone or in the absence of activator (control). The proteolytic activity of the human plasmin preparations are expressed in casein units (Cas. U.) (25, 26).

1) Casein proteolysis by a modification (25) of the method of Remmert and Cohen (26). In using this assay for plasma plasminogen, advantage was taken of the observation that acidification of human plasma to pH 2.0 destroys a considerable portion of the antiplasmin activity and yields aproximately a threefold increase in proteolytic activity upon the addition of streptokinase (23). Therefore, all plasmas to be assayed by this method first were adjusted to pH 2.0 by the addition of one-sixth N HCl, allowed to stand for 15 minutes, and then reneutralized with one-sixth N NaOH.

2) Hydrolysis of benzoyl-l-arginine methyl ester (BAMe). Based on the study of Schwert and Takenaka (29), a simple spectrophotometric assay for the hydrolysis of BAMe was devised which depends on the absorption difference between benzoyl arginine and BAMe at 2,580 Å. Preliminary observations revealed that the method of Schwert and Takenaka could be readily applied to the assay of plasminogen in plasma and the technique finally developed was as follows[6]:

[6] The detailed observations upon which this technique was developed will form the basis of a separate report.

Two-tenths ml. of 1:5 dilution of plasma in 0.05 M phosphate buffer, pH 7.6, was added to 0.2 ml. of a 6,000 unit per ml. streptokinase solution in buffer. After two minutes incubation at 37° C., 6 ml. of 0.5 mg. per ml. BAMe in buffer was added and a 3 ml. aliquot immediately withdrawn into a tube containing 0.1 ml. 10 per cent acetic acid, which lowers the pH to 5.0 and stops the enzymatic reaction. After incubating the remainder at 37° C. for one-half hour, a second 3 ml. aliquot was withdrawn into 0.1 ml. 10 per cent acetic acid. The optical density of the 30 minute aliquot was read against the zero specimen at 2,580 Å. The spontaneous BAMe esterase activity of the plasma specimen was simultaneously determined by substituting 0.2 ml. buffer for the streptokinase solution in the above method. The plasminogen activity was calculated by subtracting the spontaneous activity from the total activity observed in the streptokinase sample. The results were expressed in units per ml., with one unit representing 1 μM of substrate hydrolyzed per minute under the conditions of the test (30). With this assay, normal plasma contains $65 \pm 15 \times 10^{-3}$ units per ml. plasma.

b) *Plasmin.* Plasmin was assayed by measuring the proteolytic activity of normal and previously acidified plasma on casein. Casein proteolysis was assayed by a modification (25) of the technique of Remmert and Cohen (26).

Observations were also made of the spontaneous BAMe esterase activity of plasma as noted in the previous section. Although the normally observed spontaneous activity cannot be attributed to plasmin alone, since a number of other serum enzymes are also capable of hydrolyzing BAMe, the appearance of increased amounts of plasmin in the circulation would be expected to increase the level of this plasma activity.

c) *Anti-plasmin.* The inhibition of spontaneously activated human plasmin by plasma was used as a measure of anti-plasmin activity. Plasmin was prepared as described by Alkjaersig, Fletcher and Sherry (28). Eight-tenths ml. aliquots of a purified plasmin preparation containing 33.3 casein units per ml. (57 casein units per mg. tyrosine) diluted 1:5 with 0.1 M phosphate buffer, pH 7.6, was incubated at room temperature for 15 minutes with 0.2 ml. phosphate buffer or 0.2 ml. of the plasma to be assayed. The proteolytic activity upon casein was measured and expressed as per cent-inhibition by plasma of the plasmin specimen. This method, although not a precise measure of anti-plasmin, was found to give reproducible results in these studies.

d) *Fibrinogen.* The fibrinogen content of plasma and euglobulin samples was assayed by the method of Ratnoff and Menzie (31).

IV. Inhibition studies with epsilon amino caproic acid. The demonstration that epsilon amino caproic acid is a strong competitive inhibitor in the activation of plasminogen by streptokinase, urokinase and tissue kinases and is a much weaker inhibitor of plasmin (25) afforded an opportunity to investigate whether the enhanced fibrinolytic activity seen in the plasma of patients was due to

the presence of preformed plasmin or to the presence of a plasminogen activator (*i.e.*, with activation of clot plasminogen). Figure 2 cites observations on the effect of a final concentration of 10^{-4} M epsilon amino caproic acid on the fibrinolytic activity observed with an activator alone (urokinase) and with spontaneously activated human plasmin. The test system consisted of 0.5 ml. 0.3 per cent bovine fibrinogen (Armour), 0.1 ml. of urokinase or plasmin, 0.1 ml. 0.001 M epsilon amino caproic acid (0.1 ml. buffer alone used for controls) and 0.3 ml. thrombin, 4 μ per ml. All solutions were made up in 0.01 M veronal buffer, pH 7.4. Note the lack of significant inhibition of plasmin by this concentration of epsilon amino caproic acid and the significant inhibition (approximately 30 per cent) observed with the activator preparation. The test system employed for plasma study was as follows: 0.5 ml. of reconstituted plasma euglobulin solution, 0.1 ml. of buffer or 0.001 M epsilon amino caproic acid; and 0.4 ml. of thrombin, 4 μ per ml. (all solutions in 0.01 M veronal buffer, pH 7.4.). The clots were incubated at 37° C., the lysis time was recorded and expressed in fibrinolytic units.

Experimental studies

Electroshock therapy. Samples were taken from 70 patients undergoing therapeutic electroshock.[7] Patients routinely received premedication with 0.4 mg. atropine and 400 mg. meprobamate. Venous samples were collected just before and within one minute after the convulsion ensuing from electroshock.

Pyrogens. Studies were made on five male patients receiving intravenous pyrogens, four of whom were given 0.2 γ of pyrexal (Wander), and one who received 30 million units of triple typhoid vaccine. Blood specimens were drawn before the injection and at one and two hour intervals afterwards. All patients experienced a temperature elevation of from 1.0 to 2.2° F. (rectal) during the second hour after injection and this elevation was associated with a "chill" in all cases but one.

Epinephrine. Thirteen patients were studied in the following manner: A control venous sample was taken, then 0.5 to 0.6 ml. of 1:1,000 epinephrine was given subcutaneously. Fifteen minutes later a second venous sample was drawn. In most patients, this amount of epinephrine was sufficient to produce an increase in pulse rate of 15 to 25 per minute.

Acetylcholine (AcCh). The effect of 50 mg. of intravenous AcCh was studied in two dogs. After "control" venous samples were drawn, 50 mg. of AcCh in 1 ml. water was rapidly injected. This resulted in hyperpnea, marked salivation, spontaneous defecation and urination within one minute after injection. Venous samples were drawn five minutes after injection.

In an attempt to determine whether acetylcholine may locally stimulate the production of fibrinolytic activity in veins, the following experiment was carried out on 15 patients. After a control venous sample was drawn, blood pressure cuffs were applied above each elbow and inflated to diastolic pressure. One hundred μg. of acetylcholine in 0.1 ml. saline was then injected either into or alongside of an antecubital vein of one arm. Two minutes

[7] At the Malcolm Bliss Mental Health Center. We are indebted to Drs. George Ulett and Kathleen Smith for the opportunity to study these patients.

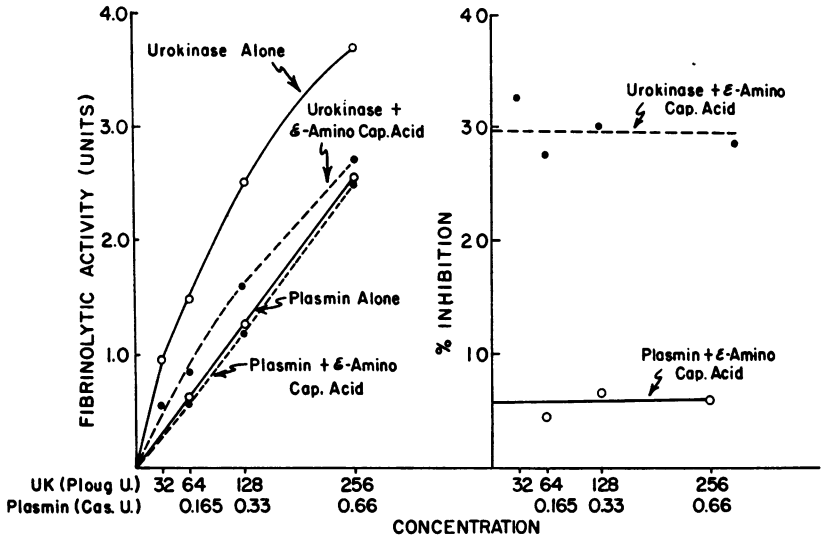

FIG. 2. INHIBITION BY EPSILON AMINO CAPROIC ACID (10^{-4} M) OF THE FIBRINOLYTIC ACTIVITY OBSERVED WITH UROKINASE AND HUMAN PLASMIN IN A BOVINE FIBRINOGEN-THROMBIN CLOT SYSTEM (LEFT PANEL)

Right panel: Results expressed as per cent inhibition of initial activity. Concentrations of urokinase and human plasmin plotted along abscissa.

TABLE I

Results of several fibrinolytic assays, antiplasmin activity and plasma euglobulin fibrinogen concentration immediately before and one minute after electroshock therapy

	Fibrinolytic assays											
	Whole blood clot lysis time		Plasma euglobulin clot lysis time		Fearnley plasma clot lysis*		Lysis of I^{131} trace-labeled human plasma clots by plasma euglobulin		Anti-plasmin activity		Fibrinogen conc. plasma euglobulin	
Patient	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
no.	*hours*		*minutes*				*cpm × 128 in supernatant*		*% plasmin inhibited*		*mg. %*	
21	>24	2	300	30	————	———+			69	72	78	57
22	>24	4	>300	50	————	+++++			66	56	72	78
23	>24	4	>300	50	————	+++++			73	66	85	79
24	>24	4	>300	130	————	+++++			59	59	90	94
25	>24	20	>300	50	————	−+++			60	69	100	90
26	>24	4	>300	45	————	————			66	69	72	67
27	>24	1.5	240	15	———	+++++			56	66	113	95
28	>24	1	>300	10	————	+++++			63	64	102	98
29	>24	>24	>300	240	————	————			65	69	97	110
30	>24	4	>300	50	————	+++++			59	60	108	92
31			300	20	————	+++++	23.4	232.8				
32			>300	20	————	+++++	25.4	170.0				
33			>300	20	————	+++++	30.3	164.4				
34			>300	20	————	+++++	22.8	198.4				

* + = Complete lysis; − = incomplete lysis. Results are shown for plasma dilutions of 1:4, 1:8, 1:16 and 1:32, respectively. Readings were made after 300 minutes of incubation at 37° C.

later, simultaneous venous samples were drawn from the "injected" vein, a wrist vein of the injected arm, and from an antecubital vein of the opposite arm. The pressure in the cuffs was then released.

Ischemia. Four types of experiments were performed, all on hospitalized patients.

Experiment 1. Venous occlusion alone (10 patients). After a control venous sample was drawn, a blood pressure cuff was applied above the elbow and inflated to diastolic pressure. After five minutes a specimen was taken from the antecubital vein and another, simultaneously, from the opposite, normal arm.

Experiment 2. Arterial occlusion, right arm; venous occlusion, left arm (10 patients). After a control venous sample was drawn, blood pressure cuffs were applied above the elbow to both arms. The right arm was made ischemic by a pressure of 50 mm. Hg above systolic pressure, and the left arm was occluded at diastolic pressure. After five minutes, simultaneous venous samples were drawn from each arm.

Experiments 3. Ischemia at wrist; venous occlusion at elbow (right), and venous occlusion (left) (10 patients). A control venous sample was drawn. The right wrist was then made ischemic with a blood pressure cuff applied at the wrist at a pressure 50 mm. above systolic pressure. Blood pressure cuffs were then placed above the elbow on each arm and inflated to diastolic pressure. After five minutes, simultaneous venous samples were taken from an antecubital vein of each arm.

Experiment 4. Ischemia followed by venous occlusion (10 patients). A control sample was drawn from the left arm. A blood pressure cuff was applied above the right elbow and maintained at 20 mm. Hg greater than systolic pressure for 10 minutes, and then lowered to diastolic pressure for another five minutes. A venous sample was then taken from the right arm and from the opposite, nonoccluded control arm.

Exercise. Studies were made on 12 healthy young adults (nine male, three female) who underwent fairly strenuous exercise (basketball game) over a two hour period. Venous blood specimens were drawn before and at the end of the exercise period. In seven (five males, two females), additional specimens were drawn at the end of 20 minutes of exercise.

Emotion. Nine members of the house staff were subjected to a moderate emotional experience and their fibrinolytic response studied. Prior to the beginning of regular ward rounds by one of us (S. S.), a specimen of blood was collected for "some experimental work." During the conduct of the rounds, the house staff was suddenly exposed to an unsuspected harangue on their inefficiency, ineptitude, and so forth. After 30 minutes of such diatribe, the house staff was informed of the true reason for the experiment and another blood specimen withdrawn.

RESULTS

I. Observations on the production of fibrinolytic activity by various stimuli

a) Electroshock. The results obtained in 14 electroshocked patients utilizing a variety of assay procedures are shown in Table I. Note the relative convenience of the plasma euglobulin clot lysis method for studying changes in plasma fibrinolytic activity. No significant changes were found in the

plasma levels of anti-plasmin. Also, the fibrinogen content of the plasma euglobulin solutions appeared to be independent of the lysis times observed in "before" and "after" samples. Plasma and euglobulin solutions were also tested upon Astrup fibrin plates and although some lysis was produced by most "after" specimens, the areas of lysis were often incomplete and difficult to quantitate, particularly with the plasma samples.

Figure 3A illustrates the pattern of response among 42 patients receiving electroshock, as determined by plasma euglobulin clot lysis, and expressed in activity units. It can be seen that all but four patients had less than 0.1 unit in their "before" samples, whereas only one patient had less than 0.1 unit after electroshock. The average values for "before" and "after" specimens were less than 0.1 unit (*i.e.*, a lysis time greater than five hours) and 0.9 unit (*i.e.*, a lysis time of 35 minutes), respectively.

The age and sex of the patient, and the number of prior shock treatments showed no correlation with the degree of fibrinolytic activity manifested after electroshock. Samples taken from the same patients following electroshock therapy on different days demonstrated the tendency of certain patients' plasma to manifest either consistently fast or slow lysis times. For example, the "after" samples of one patient showed euglobulin clot lysis times of 30, 15, 18 and 21 minutes on four occasions, whereas those of another patient lysed in 150, 50 and 120 minutes on three occasions.

Five patients, whose convulsions were either abolished or greatly minimized by premedication with succinylcholine, all showed very striking fibrinolytic activity after electroshock, demonstrating that the muscular component of electrically induced convulsions was not required for the production of the increased fibrinolytic activity (Figure 3B).

Five patients were given 3.0 mg. atropine sulphate intravenously three to four minutes before electroshock was administered. Blood samples, drawn afterwards in the usual manner, revealed enhanced fibrinolytic activity (Figure 3C), demonstrating that at the time interval and dosage level used, atropine failed to block the appearance of fibrinolytic activity following electroshock.

Five patients receiving electroshock therapy were studied in an effort to determine whether the fibrinolytic activity found after electroshock was locally produced. After control samples were taken, a blood pressure cuff was applied to the patient's right arm and maintained at 50 mm. of Hg

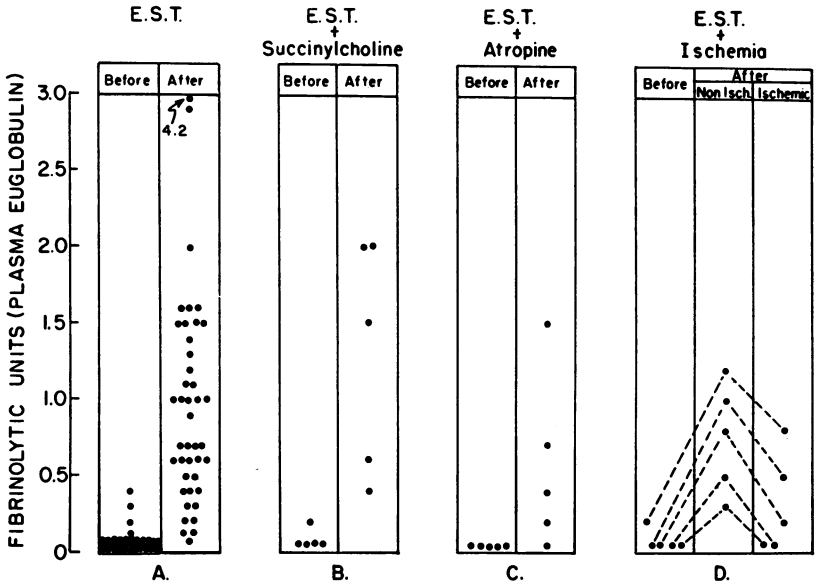

FIG. 3. APPEARANCE OF FIBRINOLYTIC ACTIVITY IN EUGLOBULIN FRACTION OF PLASMA OF PATIENTS SUBJECTED TO ELECTROSHOCK THERAPY

Fibrinolytic activity expressed in units (one unit equivalent to lysis time of 30 minutes under conditions of the test). See text for details.

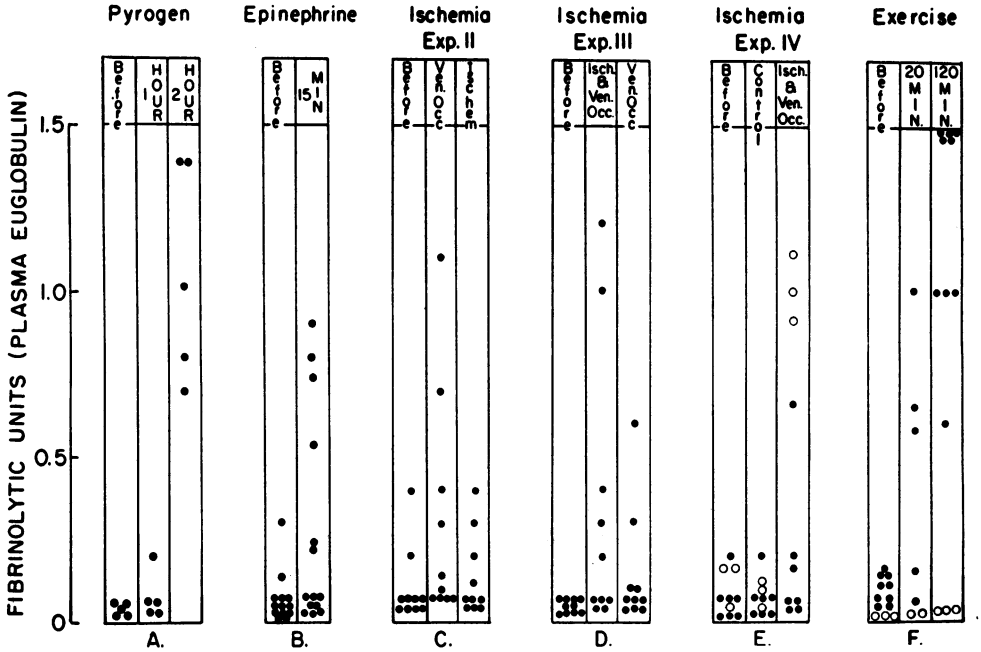

FIG. 4. APPEARANCE OF FIBRINOLYTIC ACTIVITY IN EUGLOBULIN FRACTION OF PATIENTS SUBJECTED TO PYROGENS, EPINEPHRINE, ISCHEMIA AND SEVERE EXERCISE
See text for details.

greater than systolic pressure, thereby arresting the arterial and venous flow in the limb during the convulsion, after which venous samples were taken from the ischemic arm and from the opposite, nonoccluded arm. The results shown in Figure 3D reveal that increased fibrinolytic activity was demonstrated in the blood from the ischemic arm of several of the patients, suggesting local production. However, in all cases the activity in the opposite, nonischemic arm was greater. This last observation is consistent with the view that during a period of active ischemia the appearance of fibrinolytic activity may be actually suppressed (14).

b) Pyrogens, exercise, epinephrine and ischemia. Figure 4 cites observations on the fiibrinolytic activity induced by pyrogens, exercise, epinephrine and ischemia, as measured by the plasma euglobulin clot lysis technique. Enhanced fibrinolytic activity was observed after each of these procedures, but most consistently and to a greater extent after pyrogens and intense exercise. Accelerated whole blood fibrinolysis and accelerated lysis of I^{131}-trace-labeled plasma clots was observed whenever the plasma euglobulin clot lysis time was rapid and the relative convenience of the plasma euglobulin clot lysis technique was demonstrated again.

With pyrogens (Figure 4A) the enhanced fibrinolytic activity appeared in the second hour specimen or shortly after the "chill." With exercise (Figure 4F) the most marked increase also was evident at the end of the two hour period and specimens drawn at the end of 20 minutes demonstrated a more moderate rise in fibrinolytic activity. The observations, though limited, suggest that males (solid circles) may have a greater response to exercise than do females (open circles).

Epinephrine (Figure 4B) and ischemia produced a less consistent and less striking response but enhancement of fibrinolytic activity was observed in a number of the patients. We were able to confirm the observations of Kwaan and McFadzean (14) that increased fibrinolytic activity does not occur with venous occlusion alone (Experiment 1), but does in association with ischemia where it was demonstrated in occluded venous segments of the opposite arm (Figure 4C), in occluded venous segments of the same arm, proximal to the ischemic area (Figure 4D), and in occluded venous segments draining a previously ischemic area (Figure 4E). Observations made during the latter study suggest that females (open circles) may have a more intense fibrinolytic response to ischemia than do males (solid circles).

c) Acetylcholine. Acetylcholine, when injected intravenously in large amounts into two dogs, produced an increase in circulating fibrinolytic activity. In one animal the activity increased from 0.2 to 1.8 units, and in another from 0.4 to 4.2 units. In the latter animal, whole blood fibrinolysis was complete in the postacetylcholine specimen in 100 minutes. However, 14 of 15 patients who were injected intra- or paravenously with small amounts of acetylcholine showed no increase in fibrinolytic activity and the remaining patient developed a slight activity. The same experiment was carried out using one of the authors (R. I. L.) as the subject, except that a five minute interval between the injection and the drawing of the venous samples was used. In this case, blood from the paravenously injected vein showed 1.2 fibrinolytic units compared to 0.3 unit in the "before" sample. However, no significant activity was found upon three repetitions of this experiment in the same subject.

d) Emotion. Emotional stress, under the conditions of our experiment, failed to elicit a significant increase in circulating fibrinolytic activity in any of the nine subjects studied.

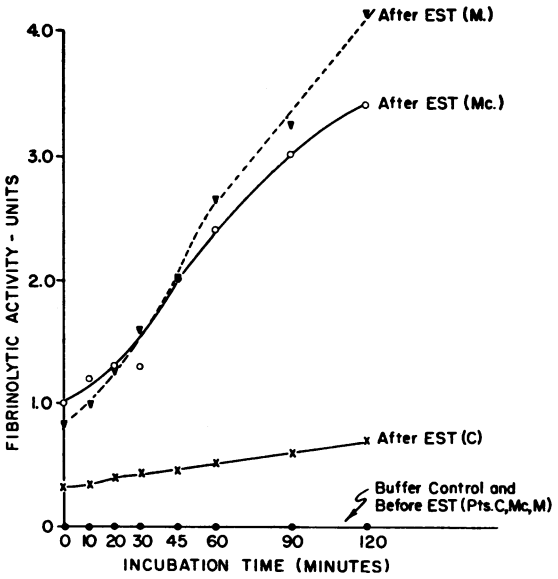

FIG. 5. ACTIVATION CURVES WITH PLASMA EUGLOBULIN OF THREE PATIENTS BEFORE AND AFTER ELECTROSHOCK THERAPY (EST)

Plasma euglobulin incubated with purified human plasminogen at 25° C. Aliquots removed at time intervals and tested for fibrinolytic activity in standard bovine fibrinogen-thrombin clot system. Fibrinolytic activity expressed in units.

II. Demonstration of activator in the plasma of patients with enhanced fibrinolytic activity

Since the lysis of preformed human plasma clots has been shown to be a sensitive index of the level of activator in the test solution and relatively insensitive to the presence of plasmin (23), the marked increase in the lysis of trace-labeled human plasma clots by the "after" specimens of patients

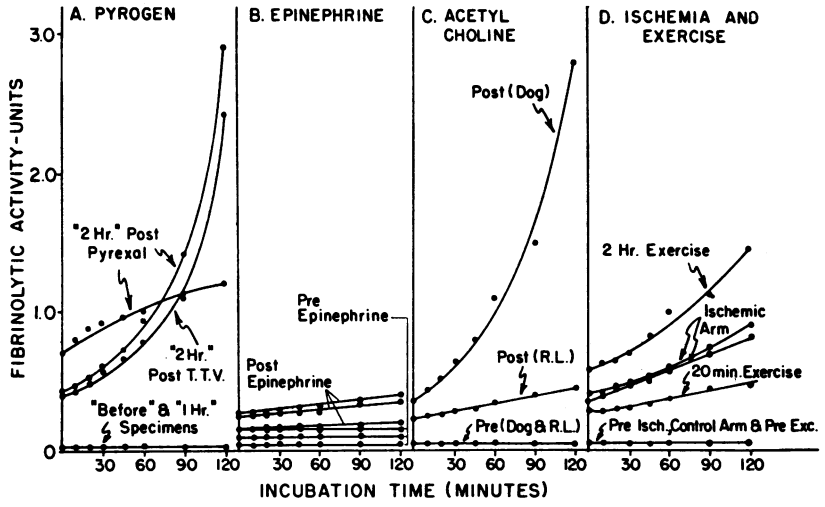

FIG. 6. ACTIVATION CURVES WITH PLASMA EUGLOBULIN OF PATIENTS SUBJECTED TO PYROGENS (A), EPINEPHRINE (B), ACETYLCHOLINE (C), ISCHEMIA AND EXERCISE (D).

Technique as previously described. See text for details.

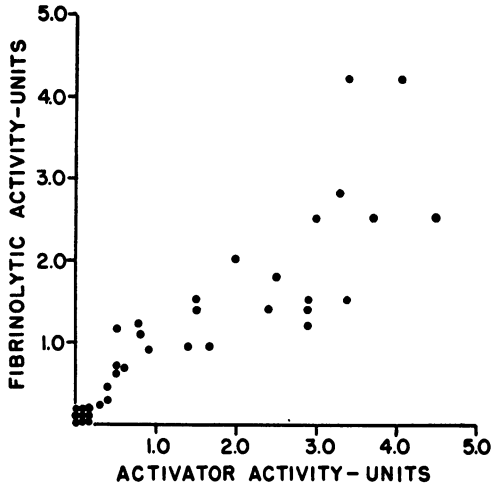

FIG. 7. CORRELATION BETWEEN THE FIBRINOLYTIC ACTIVITY OF THE PLASMA EUGLOBULIN FRACTION AND ITS CONTENT OF ACTIVATOR ACTIVITY

Fibrinolytic activity determined by the plasma euglobulin clot lysis technique. Activator activity determined by activation studies described in text and expressed in arbitrary units (one unit represents that amount of activity required to increase the fibrinolytic activity of the activation mixture by one unit in a two hour incubation period).

undergoing electroshock (Table I, Patients 31 through 34), pyrogens and exercise indicated that the increased fibrinolytic activity was due to the presence of a plasminogen activator. The presence of a plasminogen activator in the plasma of these patients was unequivocally demonstrated by activation experiments utilizing the patients' plasma euglobulin as a source of activator. Figure 5 demonstrates the presence of a plasminogen activator in the euglobulin fraction of the plasma of three patients immediately following electroshock.

Similar observations were made following pyrogens and exercise and in those patients with enhanced fibrinolytic activity following epinephrine, acetylcholine and ischemia (Figure 6).

Precise quantitation of the amount of activator present was not attempted since some of the activation curves (Figure 6A and C) differed from that observed with urokinase, raising considerations requiring further study. However, more activator was present in those patients with the highest fibrinolytic activity, and in general a correlation existed between the amount of activator observed and the fibrinolytic activity determined by the plasma euglobulin clot lysis technique (Figure 7). Thus in specimens with the highest fibrinolytic activity (electroshock, pyrogens and severe exercise) very significant amounts of activator were present whereas with specimens of less intense fibrinolytic activity (epinephrine and ischemia) smaller amounts of activator were present. Figure 6C demonstrates the appearance of a relatively large amount of activator in the plasma of a dog who developed 4.2 units of fibrinolytic activity following 50 mg. of acetylcholine intravenously and a much smaller amount of activator in the plasma of R. I. L. who developed a more moderate increase in fibrinolytic activity (1.2 units) following a paravenous injection of 100 μg. of acetylcholine. No activator could be demonstrated in the plasma specimens of those individuals in whom enhanced fibrinolytic activity did not develop following the various stimuli used.

III. Lack of significant changes in other constituents of the plasminogen system

The plasma levels of fibrinogen, plasminogen and anti-plasmin were studied in a number of patients who developed enhanced fibrinolytic activity following the various stresses described, but no significant changes in these levels as compared to the control observations were noted, nor could any increase in plasmin activity be demonstrated by the techniques employed (casein proteolysis and spontaneous BAMe esterase activity).

IV. Inhibition of enhanced fibrinolytic activity by an inhibitor of plasminogen activation

Since the previous observations suggested that the enhanced fibrinolytic activity was a reflection of the activator concentration rather than any preformed plasmin, this probability was investigated further by means of an activator inhibitor. The plasma euglobulins from nine electroshocked patients were prepared, and the clot lysis time determined in the presence and absence of a 10^{-4} M concentration of epsilon amino caproic acid. The data in Table II demonstrate that the epsilon amino caproic acid exerted a significant inhibitory effect which averaged 39 per cent. This observation, in good agreement with the 30 per cent inhibition observed with urokinase at a similar epsilon amino caproic acid concentration, but significantly

greater than the 4 to 6 per cent noted with plasmin (see Methods section), indicates that the observed fibrinolytic activity reflected the activator concentration rather than preformed plasmin.

DISCUSSION

Enhanced fibrinolytic activity has been demonstrated to occur in the blood of most patients subjected to therapeutic electroshock, pyrogen injections, intense exercise and in dogs given large intravenous doses of acetylcholine. A less consistent fibrinolytic response has been observed to occur among those patients given subcutaneous epinephrine or those subjected to ischemia. No fibrinolytic response was observed after a moderately distasteful emotional experience. The observations of Kwaan, Lo and McFadzean (13), that local paravenous or intravenous acetylcholine consistently produces increased fibrinolytic activity, were not confirmed in these studies.

The mechanism underlying the appearance of marked fibrinolytic activity has been demonstrated, in all the above circumstances, to involve the appearance in the blood of an activator of human plasminogen. Evidence for the presence of a plasminogen activator was furnished both by experiments demonstrating activation of purified human plasminogen and by the results obtained from studies utilizing I^{131}-tagged human plasma clots, the lysis of which has been shown to be a sensitive indicator of the presence of activator in the test solution. Furthermore, the plasma levels of anti-plasmin activity were demonstrated to remain unchanged in samples showing marked fibrinolytic activity. The plasminogen and fibrinogen levels of control and experimental samples, when assayed, were also found to show no consistent change.

The presence of enhanced fibrinolytic activity in plasma need not necessarily be construed as indicating the presence of free plasmin in the circulation. Although fibrinolysis is produced by the action of plasmin, fibrinolytic activity may result either from the presence of preformed plasmin or from the presence of a plasminogen activator which, by virtue of its ability to activate the plasminogen adsorbed onto fibrin during clot formation, also is capable of mediating fibrinolysis. Müllertz (32) recently has reviewed the evidence

TABLE II

Effect of epsilon amino caproic acid on plasma euglobulin clot lysis time following electroshock

Patient	Euglobin clot lysis time		Fibrinolytic activity		Inhibition
	Without EACA	With EACA*	Without EACA	With EACA	
no.	*minutes*		*units*		%
1	63	98	0.49	0.30	38
2	43	70	0.68	0.42	38
3	58	75	0.50	0.39	22
4	45	75	0.66	0.39	41
5	60	115	0.50	0.26	48
6	30	65	1.00	0.46	54
7	30	43	1.00	0.68	32
8	47	85	0.63	0.35	44
9	69	125	0.43	0.27	37

* Epsilon amino caproic acid; 10^{-4} M concentration in test.

which suggests that circulating fibrinolytic activity may be a measure of the presence of plasminogen activator. The observations cited in this article of enhanced fibrinolytic activity occurring in the presence of a plasminogen activator, yet in the absence of demonstrable plasmin or significant changes in the plasminogen or fibrinogen levels, indicate that the increased fibrinolytic activity in our patients was due to the activation of plasminogen after clot formation had occurred rather than to increased levels of circulating plasmin. Additional support for this view is derived from the studies with epsilon amino caproic acid (an inhibitor of plasminogen activation) in which inhibition of the enhanced fibrinolytic activity following electroshock was observed. The findings of Schultz, Moorman, Matoush and Lincoln (33), who were unable to demonstrate increased levels of plasma esterase activity for tosyl arginine methyl ester and lysine ethyl ester in bloods with marked fibrinolytic activity obtained from patients who had received pyrogen injections, are also consistent with this interpretation.

The view that increased fibrinolytic activity is associated with the presence of a plasminogen activator *per se* is also in accord with the evidence recently accumulated by Sherry, Fletcher and Alkjaersig (34) concerning the mechanism of thrombolysis *in vivo*. Under this scheme, the circulating level of plasminogen activator rather than the level of free plasmin is the critical factor determining thrombolysis. Small amounts of activator in the circulation ordinarily result in no measur-

able free plasmin because of the strong anti-plasmin activity of plasma. However, this same amount of activator will produce thrombolysis and to explain this observation it has been suggested that the activator has the ability to diffuse into or adsorb onto the clot and activate the intrinsic clot plasminogen in a relatively inhibitor-free environment. The failure to observe a decrease in plasminogen in plasmas containing an activator may be attributed either to the insensitivity of the assay methods to small changes in plasminogen levels, or to the existence in the circulation of a physiologic mechanism for inhibiting the activator (a mechanism not operative inside the clot). The demonstrated "lability" of the activator in plasma lends some credence to the presence of an inhibitory mechanism.

The demonstration that a plasminogen activator appears in the blood of patients following a wide variety of stimuli (electroshock, pyrogen, exercise, ischemia, epinephrine and acetylcholine) extends the observations of others who have noted the appearance of an activator under select situations. Thus, Müllertz (35) demonstrated the presence of an activator in the spontaneously lytic blood obtained from human subjects after death from anoxia and in patients following electroshock. Albrechtsen, Storm and Trolle (16) describe the presence of both free plasmin and an activator of plasminogen in the blood of a woman dying with amniotic fluid embolism; and Von Kaulla (12), from his studies, considers that the fibrinolytic activity seen after pyrogen injections is most likely the result of the release from the tissues of an activator of plasminogen. The presence of some fibrinolytic activity in all blood specimens has been known for a long time and this phenomenon may be magnified by diluting the plasma (8) or by utilizing the plasma euglobulin clot lysis technique. These observations suggest the possibility that there are trace levels of activator present in the circulation under normal conditions.

Although our studies indicate the significance of activator under physiological circumstances, they should not be construed as indicating that all changes in fibrinolytic activity reflect changes in activator concentration alone. Changes in plasma inhibitors may influence the level of fibrinolytic activity observed. Thus it has been suggested that bishydroxycoumarin and its derivative decrease fibrinolytic activity by increasing the anti-plasmin concentration (36), though it is not apparent from the methods used whether this decrease is due to an increase in anti-plasmin or to an activator inhibitor. Furthermore, the release of large amounts of activator particularly in the presence of reduced plasma inhibition may account for those instances where increased fibrinolytic activity has been associated with changes in fibrinogen, plasminogen and other components of the coagulation mechanism.

Little is known of the nature of the plasminogen activator found in these studies. It is apparently adsorbed onto fibrin during the process of clotting and can be precipitated with the plasma euglobulin fraction. Its lability is suggested by the slowly decreasing fibrinolytic activity of plasma samples stored at $-20°$ C. and its more rapid disappearance at higher temperatures (21, 37). It is an attractive hypothesis that the activator observed in the above studies is the same material in all cases and like other plasminogen activators (38) will prove to be a proteolytic enzyme capable of splitting arginine and lysine esters, but we have no proof for this.

The possibility that a neurogenic mechanism might mediate the production of fibrinolytic activity has been suggested by Kwaan, Lo and McFadzean (13), and is supported by certain of the experimental data in this report. The sudden, local production of fibrinolytic activity in an arm in which the arterial and venous flow had been arrested during electroshock suggests that this response may have been mediated through the nervous system. It is well recognized that patients undergoing electroshock experience a generalized autonomic discharge. Considering the magnitude of this discharge, the failure of atropine as employed in these experiments to block the response to electroshock does not preclude the possibility of the existence of a cholinergic effector-mechanism. Furthermore, both the demonstration of fibrinolytic activity following intravenous acetylcholine in the dog and the similarity of the associated autonomic response to that manifested by patients receiving electroshock, suggest that a cholinergic discharge may mediate the fibrinolytic effect seen with an electroshock. Our ability to confirm the observation of reflex stimulation of fibrinolytic activity (see Ischemia, Experiment 2)

lends additional support to the presence of a neurogenic mechanism.

It has been suggested that fibrinolytic activity is imparted to the blood by the walls of veins (13) since blood withdrawn from superficial veins of an occluded arm may, after various stimuli, contain enhanced fibrinolytic activity. However, in studies currently in progress here employing isotopically-labeled albumin, it has been shown that such "venous" blood consists, in a large degree, of blood recently arrived from the arterial tree; and that it is, therefore, primarily not blood that has been contained within the veins during the period of occlusion. Moreover, data obtained recently from related experiments with ischemia have suggested that, following a period of ischemia, the greatest concentration of fibrinolytic activity is to be found in the fresh blood that perfuses the ischemic limb upon release of the cuff. These findings are consistent with the view that the capillary bed may even be a more important site from which fibrinolytic activity is imparted to the blood.

Although Macfarlane and Biggs have stressed the importance of emotional stress in stimulating fibrinolytic activity (4), it is of interest that not all emotional stress is accompanied by increased fibrinolytic activity. The stress utilized in our study, though real, was neither frightening nor life threatening and, for this reason, may have failed to elicit an increase in activity.

Evidence is accumulating that fibrinolysis is a fundamental physiologic process and not merely a phenomenon limited to certain pathologic states. Fearnley and Lackner (8) have reported low levels of fibrinolytic activity in some healthy, nonstressed adults and we have observed the presence of small amounts of fibrinolytic activity in a number of our patients' "control" blood specimens. In recent articles, Astrup (39) and Duguid (40) have speculated upon the possible relationship of fibrinolysis to the development of atherosclerosis. It is of interest, in this respect, that our female patients appeared to produce a greater fibrinolytic response to ischemia than did males (see Ischemia, Experiment 4.).

SUMMARY

1. Enhanced fibrinolytic activity has been demonstrated to occur in the blood of almost all patients subjected to electroshock and pyrogen injections and in male subjects following severe exercise. A less consistent response has been shown to occur following epinephrine, acetylcholine and ischemia. The plasma euglobulin clot lysis time appears to be a simple and sensitive technique for assaying plasma fibrinolytic activity.

2. The mechanism underlying the appearance of enhanced fibrinolytic activity under the circumstances described has been demonstrated to involve the appearance in the blood of a plasminogen activator.

3. Detailed biochemical studies on plasma of these patients failed to reveal evidence of increased amounts of plasmin or decreased plasminogen or anti-plasmin levels. It is suggested on the basis of the evidence presented that the enhanced fibrinolytic activity was produced by the direct activation of intrinsic clot plasminogen rather than increased amounts of circulating plasmin.

4. Evidence is presented which suggests that plasminogen activators may be locally produced by a neurogenic mechanism and the possibility that the capillary bed may be an important source is discussed.

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REFERENCES

1. Macfarlane, R. G., and Biggs, R. Fibrinolysis. Its mechanism and significance. Blood 1948, **3**, 1167.
2. Nolf, P. Contribution à l'étude de la coagulation du sang, la fibrinolyse. Arch. int. Physiol. 1908, **6**, 306.
3. Macfarlane, R. G. Fibrinolysis following operation. Lancet 1937, **1**, 10.
4. Macfarlane, R. G., and Biggs, R. Observations on fibrinolysis. Spontaneous activity associated with surgical operations, trauma, etc. Lancet 1946, **2**, 862.
5. Biggs, R., Macfarlane, R. G., and Pilling, J. Observations on fibrinolysis. Experimental activity produced by exercise or adrenaline. Lancet 1947, **1**, 402.
6. Yudin, S. S. Transfusion of cadaver blood. J. Amer. med. Ass. 1936, **106**, 997.
7. Mole, R. H. Fibrinolysin and the fluidity of the blood post-mortem. J. Path. Bact. 1948, **60**, 413.
8. Fearnley, G. R., and Lackner, R. The fibrinolytic activity of normal blood. Brit. J. Haemat. 1955, **1**, 189.

9. Soulier, J. P., and Koupernik, C. Constance de la fibrinolyse au cours du choc acétyl-cholinique. Sang 1948, **19**, 362.
10. Fantl, P., and Simon, S. E. Fibrinolysis following electrically induced convulsions. Aust. J. exp. Biol. med. Sci. 1948, **26**, 521.
11. Tagnon, H. J., Levenson, S. M., Davidson, C. S., and Taylor, F. H. L. The occurrence of fibrinolysis in shock, with observations on the prothrombin time and the plasma fibrinogen during hemorrhagic shock. Amer. J. med. Sci. 1946, **211**, 88.
12. Von Kaulla, K. N. Intravenous protein-free pyrogen. A powerful fibrinolytic agent in man. Circulation 1958, **17**, 187.
13. Kwaan, H. C., Lo, R., and McFadzean, A. J. S. On the production of plasma fibrinolytic activity within veins. Clin. Sci. 1957, **16**, 241.
14. Kwaan, H. C., and McFadzean, A. J. S. On plasma fibrinolytic activity induced by ischaemia. Clin. Sci. 1956, **15**, 245.
15. Kwaan, H. C., McFadzean, A. J. S., and Cook, J. On plasma fibrinolytic activity in cryptogenic splenomegaly. Scot. med. J. 1957, **2**, 137.
16. Albrechtsen, O. K., Storm, O., and Trolle, D. Fibrinolytic activity in the circulating blood following amniotic fluid infusion. Acta haemat. (Basel) 1955, **14**, 309.
17. Sherry, S., and Alkjaersig, N. Studies on the fibrinolytic enzyme of human plasma. Thromb. Diath. Haem. 1957, **1**, 264.
18. Astrup, T. Fibrinolysis in the organism. Blood 1956, **11**, 781.
19. Medart, W. S. Effect of divalent cations on proteolysis of fibrinogen. Proc. Soc. exp. Biol. (N. Y.) 1958, **97**, 728.
20. Milstone, H. A factor in normal human blood which participates in streptococcal fibrinolysis. J. Immunol. 1941, **42**, 109.
21. Fearnley, G. R., Revill, R., and Tweed, J. M. Observations on the inactivation of fibrinolytic activity in shed blood. Clin. Sci. 1952, **11**, 309.
22. Astrup, T., and Müllertz, S. The fibrin plate method for estimating fibrinolytic activity. Arch. Biochem. 1952, **40**, 346.
23. Alkjaersig, N., Fletcher, A. P., and Sherry, S. The mechanism of clot dissolution by plasmin. J. clin. Invest. **In press.**
24. Kline, D. L. The purification and crystallization of plasminogen (profibrinolysin). J. biol. Chem. 1953, **204**, 949.
25. Alkjaersig, N., Fletcher, A. P., and Sherry, S. Epsilon amino caproic acid, an inhibitor of plasminogen activation. J. biol. Chem. In press.
26. Remmert, L. F., and Cohen, P. P. Partial purification and properties of a proteolytic enzyme of human serum. J. biol. Chem. 1949, **181**, 431.
27. Ploug, J., and Kjeldgaard, N. O. Urokinase. An activator of plasminogen from human urine. I. Isolation and properties. Biochim. biophys. Acta 1957, **24**, 278.
28. Alkjaersig, N., Fletcher, A. P., and Sherry, S. The activation of human plasminogen. I. Spontaneous activation in glycerol. J. biol. Chem. 1958, **233**, 81.
29. Schwert, G. W., and Takenaka, Y. Spectrophotometric determination of trypsin and chymotrypsin. Biochim. biophys. Acta 1955, **16**, 570.
30. Committee on Biological Chemistry of the Division of Chemistry and Chemical Technology, National Academy of Sciences—National Research Council. International unit of enzyme activity. Science 1958, **128**, 19.
31. Ratnoff, O. D., and Menzie, C. A new method for the determination of fibrinogen in small samples of plasma. J. Lab. clin. Med. 1951, **37**, 316.
32. Müllertz, S. Activation of plasminogen. Ann. N. Y. Acad. Sci. 1957, **68** (1), 38.
33. Schultz, R. L., Moorman, J. A., Matoush, L. O., and Lincoln, A. F. Evaluation of synthetic substrate methods for clinical determinations of fibrinolysis. Proc. Soc. exp. Biol. (N. Y.) 1957, **94**, 198.
34. Sherry, S., Fletcher, A. P., and Alkjaersig, N. The fibrinolysin system: Some physiological considerations *in* Connective Tissue, and Atherosclerosis, I. Page, Ed. New York, Academic Press Inc., 1959. In press.
35. Müllertz, S. A plasminogen activator in spontaneously active human blood. Proc. Soc. exp. Biol. (N. Y.) 1953, **82**, 291.
36. Niewiarowski, M., and Wegrzynowicz, Z. Der Einfluss von Dikumarol-derivaten auf das fibrinolytische System im Plasma. Abstracts of communications, Fourth International Congress of Biochemistry, Vienna, 1958, p. 163.
37. Truelove, S. C. The lability of human fibrinolysin. Clin. Sci. 1953, **12**, 75.
38. Alkjaersig, N., Fletcher, A. P., and Sherry, S. The activation of human plasminogen. II. A kinetic study of activation with trypsin, urokinase, and streptokinase. J. biol. Chem. 1958, **233**, 86.
39. Astrup, T. The biological significance of fibrinolysis. Lancet 1956, **271**, 565.
40. Duguid, J. B. A concept of the pathogenesis of atherosclerosis *in* Connective Tissue, and Atherosclerosis, I. Page, Ed. New York, Academic Press Inc., 1959. In press.