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STUDIES ON THE THROMBOLYTIC ACTIVITY OF HUMAN PLASMA *

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The process of *in vivo* clot dissolution, termed thrombolysis,¹ is complex, involving the interaction of clot components with the surrounding plasma. Participating in this interaction are plasminogen, plasminogen activator, plasmin, the plasmin inhibitory activity of plasma, and fibrin (1). Although much is known of the nature and quantities of individual components, the complexity of the thrombolytic process has led to difficulty in assessment of the overall capability of the plasma for effecting clot lysis under physiological conditions—an activity hereafter termed plasma thrombolytic activity. Plasma thrombolytic activity is a property of major physiological significance (2, 3) but hitherto the technical methods used for its assay have been imperfect. Assays involving determination of whole blood or plasma clot lysis time are valuable for detecting markedly enhanced thrombolysis but are inadequate for measurement of low levels of thrombolytic activity due to the very long lysis times found and the lack of a precise endpoint. Other assays have involved physicochemical alterations of the plasma such as dilution (4, 5), isolation of the euglobulin fraction (6, 7) and extraction with organic solvents (8). Such physicochemical procedures isolate or affect to a variable degree one or more components of the thrombolytic process and, although valuable for specialized purposes, disturb the complex balance between the individual plasma components (1). Consequently, assays of this nature are unsuitable for other than qualitative determination of plasma thrombolytic activity.

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¹ The term thrombolysis is used to designate a process by which preformed clots are lysed, and its use does not imply specific reference to pathological states (1).

In order to circumvent these limitations, a sensitive measure of plasma thrombolytic activity was developed in which unaltered plasma is tested. In principle the method involves the determination of radioactivity released from isotopically-labeled human plasma clots immersed in unaltered plasma. Although an *in vitro* procedure, the use of unaltered plasma and preformed clots mimics *in vivo* conditions and permits the obtaining of physiologically relevant measurements. Using this isotopic clot assay, measurements of plasma thrombolytic activity have been made on plasma obtained from healthy adults, from adults following stress, after the administration of drugs, and from individuals with disease. The results indicate that plasma obtained from nonstressed normal adults exhibits thrombolytic activity due primarily to the presence of a plasminogen activator and that this activity varies as a result of stress and disease.

MATERIALS AND METHODS

¹²⁵I-trace-labeled bovine fibrinogen² was prepared as previously described (1).

Human plasminogen was prepared by Kline's procedure (9) from human plasma fraction III.³

Human plasmin was prepared by spontaneous activation of human plasminogen in 50 per cent glycerol (10).

Thrombin mixture consisted of Parke-Davis thrombin diluted to 10 units per ml with equal parts 0.7 per cent calcium chloride and 6 per cent dextran. The use of the latter reagents aided in the production of more consistent and stable clots.

Urokinase⁴ contained 5,100 Ploug units per milligram dry weight.

² Salt-free bovine fibrinogen containing 96 per cent thrombin-clottable nitrogen; kindly supplied by Dr. Kent Miller, New York State Institute of Health, Albany, N. Y.

³ Human plasma fraction III was obtained from Dr. T. O. Gerlough, E. R. Squibb and Sons, through the courtesy of Dr. J. N. Ashworth of the American Red Cross.

⁴ Kindly supplied by Dr. J. Ploug, Leo Pharmaceuticals, Copenhagen, Denmark.

ϵ -Aminocaproic acid⁵ was chemically pure and chromatographically homogeneous.

Buffered saline refers to 0.9 per cent sodium chloride containing 0.1 M phosphate buffer at pH 7.6.

¹²⁵I-trace-labeled human plasma clots were formed from aged human plasma (stored for at least 30 days at 4° C) enriched with human plasminogen (*vide infra*) and containing trace quantities of ¹²⁵I-labeled bovine fibrinogen. Two-tenths ml of such plasma was clotted by the addition of 0.1 ml of thrombin mixture. The clot was caused to form along the internal surface of glass tubing (7 mm OD) by rotation of the tube in a horizontal plane at approximately 350 rpm in the chuck of an electric motor. Clots formed in this fashion were small, compact and largely free of entrapped plasma, a critical feature of the technique. When formed, the clot was floated from the tubing and placed in a test tube. Clots were washed overnight by gentle agitation in buffered saline. Because this washing procedure, necessary to produce clots of sufficiently low blank radioactivity, eluted considerable plasminogen from the clots, it was necessary to enrich the original plasma with plasminogen as noted above. The physical state of dispersion at pH 7.6 of the purified plasminogen used was such that it was taken up from plasma into the clot and not washed out (1). The concentrations of ¹²⁵I fibrinogen and plasminogen added were such that the final clot contained on the average 2.5×10^{-4} μ c per μ g fibrin (128 cpm per μ g under detection conditions used, *vide infra*), 0.4 casein unit plasminogen and 500 μ g fibrin. ¹²⁵I bovine fibrin represented approximately 2 per cent of the total. Such clots, designated as plasminogen-rich, were used for all routine determinations.

Low plasminogen and plasminogen-free ¹²⁵I-labeled plasma clots were made as above except that no purified plasminogen was added. Plasminogen content was determined by previously described methods (1) modified only insofar as multiple small clots were used for each determination. Plasminogen-poor clots contained on the average 0.04 casein unit plasminogen per clot. Such clots exhibited normal sensitivity to lysis by plasmin. Clots could be rendered plasminogen-free by heating (80° C for 30 minutes); such clots were unsusceptible to lysis by plasminogen activators and their sensitivity to lysis by plasmin was somewhat reduced (1).

Thrombolytic assay with ¹²⁵I-labeled human plasma clots was performed by draining the wash solution from a clot and then incubating the latter for 2 hours at 37° C immersed in 0.2 ml of the material to be tested for thrombolytic activity. The clot was then separated by filtration under air pressure (10 p.s.i.) using medium porosity fritted glass disks. An additional 2 ml of saline was passed through the filter to minimize loss. The radioactivity of the total filtrate was determined in a well-type scintillation detector equipped with a pulse height analyzer. The emission of the isotope (0.364 MEV peak) was determined utilizing a 5 v window. Overall radiation detection efficiency under such circumstances was 25 per cent

⁵ Kindly supplied by Dr. J. Rueggsegger, Lederle Laboratories, Pearl River, N. Y.

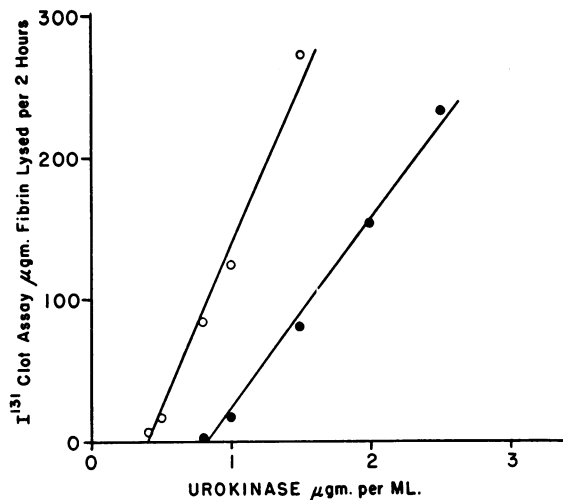


FIG. 1. LYSIS OF ¹²⁵I-LABELED HUMAN PLASMA CLOTS AS A FUNCTION OF UROKINASE CONCENTRATION. The lysis of clots was linearly related to urokinase concentration over the range illustrated. The open circles indicate results for urokinase in saline and the closed circles indicate urokinase added to aged human plasma. The intercept on the abscissa for both urokinase in saline and aged plasma indicates that the method is sensitive to the presence of less than 1 μ g urokinase per ml. The differences of the slope and intercept of the lines for urokinase in saline and in aged plasma were consistent findings.

and background was consistently in the range of 12 to 25 cpm. The use of a pulse height analyzer was considered essential since it afforded considerable increase of sample to background ratio making possible accurate detection of the low levels of radioactivity used in this technique. The statistical error of counting was less than 2 per cent. Thrombolytic activity was expressed as micrograms of fibrin lysed per 2 hours. Previous use of ¹²⁵I-labeled clots revealed residual clot nitrogen content after partial lysis to be highly correlated with residual clot radioactivity (1). Thus the radioactivity of the filtrate appeared to be a valid measure of clot lysis.

The sensitivity of the ¹²⁵I clot assay to a plasminogen activator, urokinase, is shown in Figure 1. The amount of fibrin lysed is linearly related to urokinase concentration over the range shown. Concentrations of urokinase in saline of 0.5 μ g per ml and in aged plasma of 1 μ g per ml resulted in significant lysis of the isotopically-labeled clots. The divergence between urokinase in saline and urokinase added to aged plasma was a consistent finding and presumably reflects an inhibitory effect of plasma upon urokinase. The reproducibility of the method under conditions of thrombolytic activity such as are found in normal adults was 0.81 μ g per 2 hours, as determined by the standard deviation of the difference between duplicate determinations upon 59 plasma samples (11).

Plasma euglobulin clot lysis was determined as previously described and the results expressed as units of activity (6).

Assays for plasma proteolytic activity were performed using the substrates, urea-denatured hemoglobin, as described by Anson (12) and casein, as previously described (1).

Fibrinogen was determined by the method of Ratnoff and Menzie (13).

Plasma samples were harvested from venous blood collected in oxalate tubes (4 mg potassium oxalate per ml of blood) and tested at once. Occasionally, samples were stored at 2° C for a short period prior to assay. In general, samples were obtained in the morning and in the fasting state.

RESULTS

I. Plasma thrombolytic activity of normal adults.

Upon incubation of the isotopically-labeled clots in buffered saline, radioactivity was slowly released from the clots into the surrounding media. The range and mean of 134 control observations with buffered saline are shown in Figure 2, the mean release of radioactivity being equivalent in amount to 2 μ g fibrin lysed per 2 hours. Since protein and other plasma constituents might affect the rate of such release, advantage was taken of the lability of plasma thrombolytic activity (*vide infra*) in

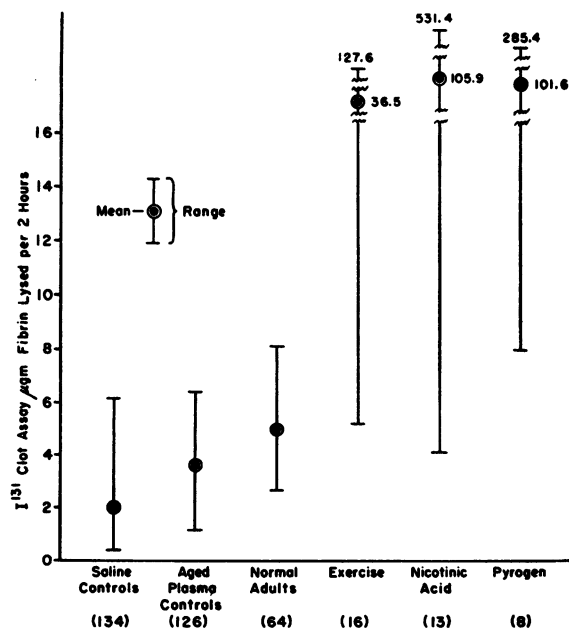


FIG. 2. PLASMA THROMBOLYTIC ACTIVITY OF NORMAL ADULTS AND ITS VARIATION FOLLOWING STRESS AND DRUG ADMINISTRATION. Figures in parentheses indicate the number of separate observations in each category. The range of observations for each category and its mean are displayed.

that aged human plasma was used as a second control. The results of 126 such tests are illustrated in Figure 2, the mean release of radioactivity being equivalent to 3.6 μ g per 2 hours. Such control determinations were made daily.

By comparison with saline, and especially with aged human plasma control values, it was possible to assess the thrombolytic activity of fresh plasma from 64 normal adults. The results are illustrated in Figure 2. The range was 2.7 to 8.1 and the mean 5.0 μ g fibrin lysed per 2 hours. Although the range of observations on normal adults overlapped that of both controls, the mean thrombolytic activity of normal adult plasma was significantly greater than either control ($p < 0.001$). These findings indicate that unaltered plasma obtained from normal individuals possesses the capacity to lyse fibrin⁶ under these *in vitro* circumstances. Presumably such plasma manifests a similar capacity under *in vivo* conditions.

No sex difference was detected, the means for males and females being 4.9 and 5.2 μ g per 2 hours, respectively. There was no correlation with age in the adult population studied. Thirteen paired morning and afternoon plasma samples provided no clear evidence of diurnal variation. Daily sampling in a small number of normal individuals disclosed no striking fluctuations of plasma thrombolytic activity.

II. *Response to exercise and to emotional stress.* Enhanced thrombolytic activity following exercise has been reported (6, 14) and the current study is confirmatory. The range and mean thrombolytic activity of plasma obtained from 16 healthy males following vigorous sustained exercise (playing basketball) are shown in Figure 2. Twelve of the 16 had plasma activity considerably in excess of normal, the mean being 7 times that for normal adults. Inappropriate timing of sample collection may have accounted for the failure to find increased plasma thrombolytic activity in 4 subjects.

Emotional stress has been implicated as a stimulus of enhanced plasma thrombolytic activity (15). Plasma samples were obtained from students before and after an examination. In-

⁶ *In vivo* thrombi and fibrinous deposits are invariably substantially contaminated with plasminogen (3) and the test system itself was likewise contaminated (see Methods section).

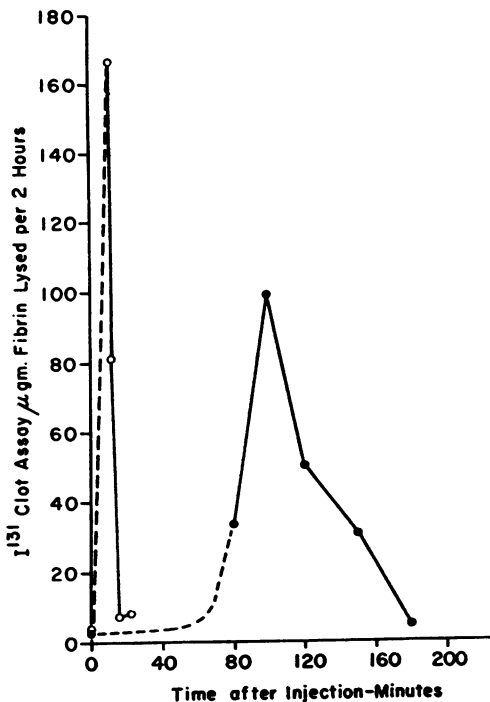


FIG. 3. COURSE OF ENHANCED PLASMA THROMBOLYTIC ACTIVITY FOLLOWING ADMINISTRATION OF NICOTINIC ACID AND PYROGEN. Time relationships of plasma thrombolytic activity to the intravenous injection of nicotinic acid (open circles) and pyrogen (closed circles) are illustrated for two separate experiments. The rise in plasma thrombolytic activity after nicotinic acid was abrupt and short-lived while that following pyrogen injection was delayed and of greater duration.

creased plasma thrombolytic activity was found in 2 of 4 subjects after the examination.

III. Drug-induced changes in plasma thrombolytic activity. Of the several drugs known to induce increased plasma thrombolytic activity, observations were made following administration of two, nicotinic acid⁷ (16) and Pyrexal⁸ (17). Nicotinic acid was given intravenously in a dose of 1 mg per kg and in all instances induced typical flushing. On 6 of 13 trials significantly increased thrombolytic activity resulted. The mean and range are shown in Figure 2. Pyrexal was given intravenously in a dose of 0.2 μg. Antipyretics

⁷ Kindly supplied by Dr. R. Herting, Abbott Laboratories, North Chicago, Ill.

⁸ A protein-free lipopolysaccharide with potent pyrogenicity prepared from the organism *Salmonella abortus equi*, kindly supplied by Dr. Schultze, Wander Company, Chicago, Ill.

were administered prior to and concurrent with pyrogen administration and at times suppressed rigor and fever customarily associated with administration of this agent. Six of 8 individuals receiving pyrogen had increased thrombolytic activity (range and mean are depicted in Figure 2). Rigor and fever were not essential for the response. Neither agent, alone or when added to plasma, altered control assay values.

Typical responses to each of these agents are shown in Figure 3. The response to nicotinic acid is prompt but short-lived. With pyrogen the increase in activity followed a variable latent period and was of greater duration. The reason or reasons for the observed failures of both agents to induce increased activity in some patients and on some occasions is not known, but such failures have been reported (17, 18).

IV. Plasma thrombolytic activity of hospitalized adults. Figure 4 illustrates the range and mean thrombolytic activity of 307 hospitalized adults. Determinations were for the most part on a single sample from each patient drawn at random times during the hospital stay. Daily fluctuations of activity, described earlier in healthy persons as small, may have influenced the findings but, as indicated by serial assays in some patients, the influence of this effect was probably unimportant. Only the major diagnosis or diagnoses were considered.

The range and mean values noted in selected disease states are depicted in Figure 4. By comparison with normal values it is apparent that increased plasma thrombolytic activity frequently was observed in association with hematologic malignancies,⁹ liver disease and acute infections. Twenty-six, 33 and 25 per cent, respectively, of the patients in these categories had levels in excess of 95 per cent confidence limits (7.4 μg per 2 hours) established from the data of normal adults. Isolated incidences of increased plasma thrombolytic activity were observed in patients suffering from sickle cell crisis, collagen diseases, myocardial infarction, cancer, diabetes mellitus, cerebral thrombosis, polycythemia vera and amyloidosis.

Decreased levels of plasma thrombolytic activity (of less certain meaning in the I¹³¹ clot assay than increased levels) were observed in patients suffer-

⁹ Including leukemia of varied cell type, lymphomas, Hodgkin's disease, reticulum cell sarcoma and multiple myeloma.

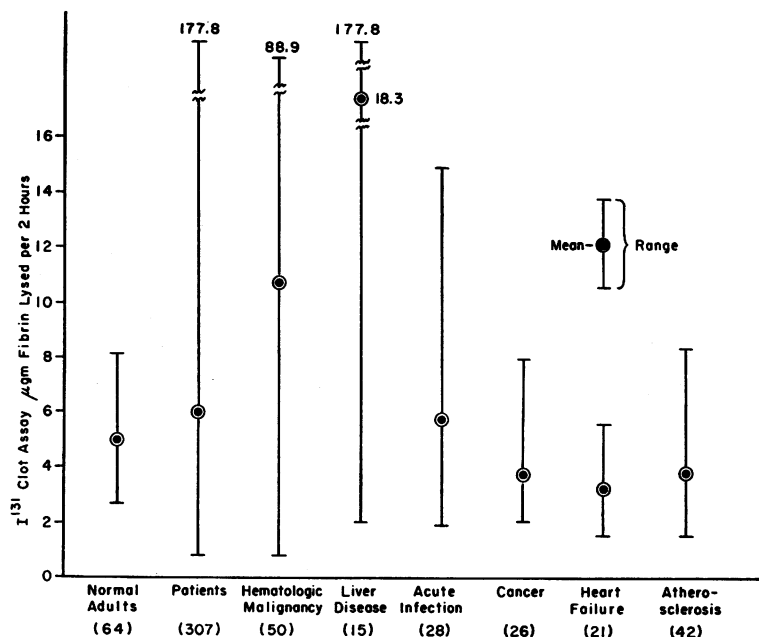


FIG. 4. PLASMA THROMBOLYTIC ACTIVITY IN SELECTED DISEASE STATES. Figures in parentheses indicate the number of separate observations in each category. The range of observations for each category and its mean are displayed. The values for normal adults are shown for comparison.

ing from a variety of disease states but most commonly among those with cancer, congestive heart failure or atherosclerosis (Figure 4).

V. Characterization of plasma thrombolytic activity. In order to define at least partially the nature of the observed thrombolytic activity of plasma, observations were made on plasma samples with respect to the following properties: a) dependence on clot plasminogen content of the test system, b) lability of the activity on storage, and c) the effect of ϵ -aminocaproic acid.

a) *Dependence on clot plasminogen concentration of the test system.* Plasma thrombolytic activity was determined on isotopically-labeled plasminogen-rich, plasminogen-poor, and plasminogen-free clots (see Methods section). Typical results appear in Table I. The thrombolytic activity was clearly a function of clot plasminogen content, being greatest with plasminogen-rich clots, less with plasminogen-poor clots and not differing significantly from control values with plasminogen-free clots. Such dependence is characteristic of plasminogen activators (1).

b) *Lability of the activity upon storage.* The thrombolytic activity of plasma was moderately

labile on storage. Serial assays revealed 50 per cent loss of activity in 1 to 3 days, which was more rapid the higher the temperature. Experiments with urokinase, a plasminogen activator, added to plasma, indicated loss of activity at a roughly similar rate. Plasmin, however, was almost immediately inactivated when added to plasma in other than large amounts, and when added in excess, a 50 per cent loss of activity occurred during a period of a few hours.

TABLE I
Effect of varying clot plasminogen concentration upon the lysis of isotopically-labeled clots immersed in test plasma*

Plasma sample	I^{131} clot assay: μ g fibrin lysed/2 hrs		
	Plasminogen-rich clot	Plasminogen-poor clot	Plasminogen-free clot
1	9.8	4.2	2.8
2	8.0	3.4	2.4
3	7.9	5.5	1.7
4	7.0	4.8	3.0
5	6.6	4.5	3.2
6	5.9	3.4	2.2

* Clot lysis was a function of clot plasminogen concentration, being greatest with plasminogen-rich clots, intermediate with plasminogen-poor clots and at or near control assay values with plasminogen-free clots.

TABLE II
Inhibition of thrombolytic activity by ϵ -aminocaproic acid

Specimen	Per cent inhibition by ϵ -aminocaproic acid		
	10^{-4} M	10^{-3} M	10^{-2} M
Plasma 1	34	47	81
Plasma 2	30	41	85
Plasma 3	25	46	80
Urokinase	23	64	85
Human plasmin	0	6	86

The results, expressed as per cent inhibition of the test system without ϵ -aminocaproic acid, illustrate the similar inhibiting effect of ϵ -aminocaproic acid on urokinase, a plasminogen activator, and on plasma thrombolytic activity; and the different effect of ϵ -aminocaproic acid on plasmin.

c) Effect of ϵ -aminocaproic acid (19). Presented in Table II are data indicating significant inhibition of plasma thrombolytic activity by ϵ -aminocaproic acid at 10^{-4} M concentration. Results are expressed as per cent inhibition of the assay value in the system without ϵ -aminocaproic acid. Urokinase was similarly inhibited. Plasmin, however, was not detectably inhibited by ϵ -aminocaproic acid until a concentration of 10^{-2} M was reached.

Moreover, the independence of thrombolytic activity and plasma proteolytic activity was apparent as thrombolytic activity was increased in plasma samples devoid of proteolytic activity, as measured with the substrates, casein and urea-denatured he-

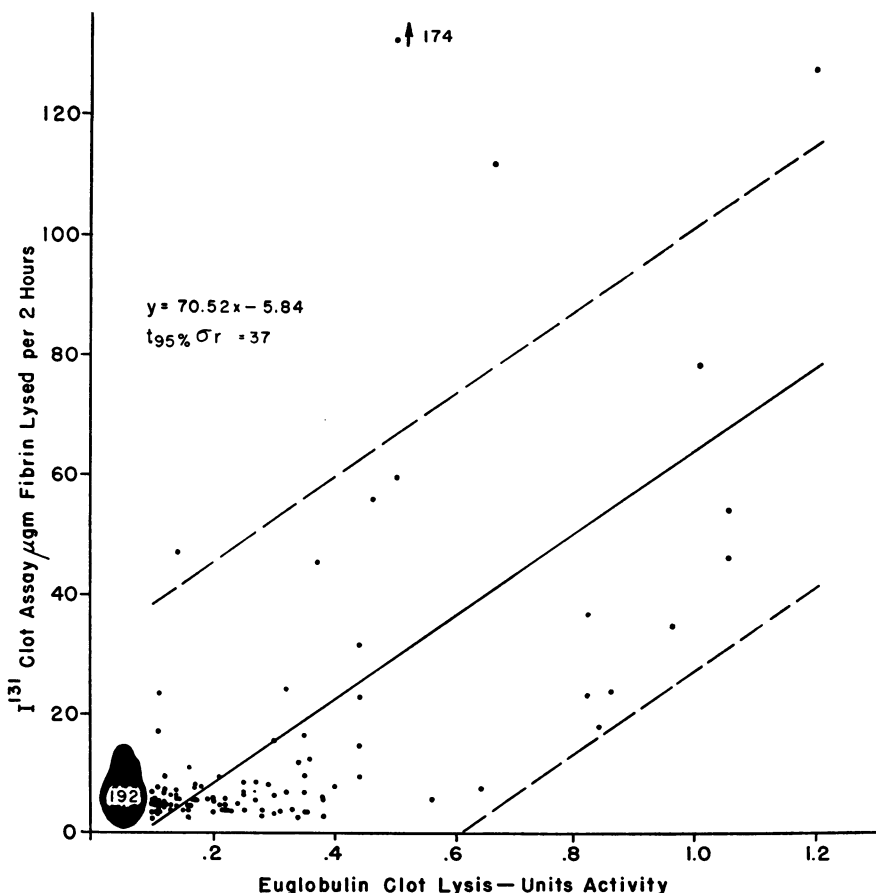


FIG. 5. CORRELATION BETWEEN THE TWO METHODS USED FOR THE DETERMINATION OF PLASMA THROMBOLYTIC ACTIVITY; ISOTOPICALLY-LABELED HUMAN PLASMA CLOT LYSIS AND EUGLOBULIN CLOT LYSIS. The results of the I^{131} clot assay are plotted against values for the euglobulin clot lysis method. The shaded area represents the results on 192 plasma samples in which the euglobulin method gave results of less than 0.1 unit (see text). These values were excluded from the statistical calculations. The regression line (solid) is shown along with the residual variance about the regression line illustrated as 95 per cent confidence limits (broken lines).

moglobin. Conversely, increased plasma proteolytic activity was at times observed in the absence of enhanced thrombolytic activity. Variations in plasma thrombolytic activity were observed without changes in plasma fibrinogen, plasminogen or antiplasmin concentrations, confirming previous data (6). On occasion when plasminogen activator concentrations were greatly increased, e.g., after drug administration, moderate falls of plasma plasminogen concentrations were recorded.

VI. Correlation of the I^{131} -labeled clot assay with plasma euglobulin clot lysis. The plasma euglobulin clot lysis test has been frequently used as a measure of overall plasma "fibrinolytic" activity (6, 7) but more recently has been shown to be at least a qualitative measure of plasma activator concentration (6). Consequently it was of interest to attempt to correlate the results of the euglobulin test with the I^{131} -labeled clot lysis assay. Both determinations were made on 302 plasma samples drawn under a variety of circumstances. The plot of results is shown in Figure 5. The euglobulin clot lysis time was expressed in units of activity. Lysis times greater than 300 minutes (customarily expressed as "normal") were recorded as less than 0.1 unit. Such values were observed in a majority of the samples, especially those in the range of normal adults as determined by the I^{131} clot method. In 110 instances the euglobulin technique resulted in values greater than 0.1 unit and it was from these samples that statistical evaluation was made. The correlation coefficient is 0.677 which is significant at the 0.001 level. However, the values for the correlation coefficient and the residual variance about the regression line (illustrated as 95 per cent confidence limits in Figure 5) indicate that the correlation, although statistically significant, is noticeably imperfect so that individual paired readings may be markedly divergent.

The significant although imprecise correlation between the isotopically-labeled human plasma clot assay and the plasma euglobulin clot lysis technique further validates the euglobulin method as a simply performed qualitative test for enhanced thrombolytic activity (6, 7). However, one of the several disadvantages of the euglobulin test is demonstrated in Figure 5 in which over half the readings positive for low levels of thrombolytic activity by the I^{131} -labeled clot method were within the "normal limits" for the euglobulin test (6, 7).

DISCUSSION

The method for measurement of thrombolytic activity used in this communication tests the capability of freshly drawn unaltered plasma to lyse a preformed clot. It closely mimics conditions *in vivo* in which unaltered plasma will pass in contact with thrombi and other fibrinous deposits. Consequently, the present observations, especially insofar as they refer to the consistent finding of thrombolytic activity in plasma from normal adults, may be regarded as being physiologically relevant to *in vivo* thrombolytic mechanisms.

Variations in levels of thrombolytic activity were demonstrated in health, following exercise, after drug administration, and in disease. Variations in health were apparently small, but after exercise, high levels of thrombolytic activity were found, an observation confirming that of others (6, 14). Similarly, the administration of nicotinic acid and pyrogens resulted in sharp rises in plasma thrombolytic activity (16, 17).

The data presented pertaining to disease, although limited, serve to focus attention on certain disease states in which altered thrombolytic activity could be of significance. The instances of increased thrombolytic activity in patients with liver disease and hematologic malignancies confirm previous reports (20-25) and the incidence suggests that increased thrombolytic activity in these states is more frequent than is commonly thought. Increased thrombolytic activity was present in some individuals with acute infections. Whether this is due to pyrexia and toxicity or is a part of the inflammatory response per se is unknown.

The suggestion of diminished thrombolytic activity in some patients suffering from cancer and congestive heart failure is noteworthy in that urokinase is excreted in diminished quantity in these conditions (26). Decreased thrombolytic activity could relate to the frequent thromboembolic complications of these states.

The evidence presented suggests that the thrombolytic activity of plasma from normal adults and from individuals with disease is due primarily to the presence of plasminogen activator. Two potential thrombolytic agents, plasmin and plasminogen activator (exemplified by urokinase) which could account for the observed activity, were com-

pared with plasma thrombolytic activity in terms of three different characteristics. In all three respects, dependence on clot plasminogen content, lability on storage, and inhibition by ϵ -aminocaproic acid in 10^{-4} M concentration, plasma thrombolytic activity resembles plasminogen activator. Thus the present data both confirm and extend to other areas recent work on *in vivo* and *in vitro* thrombolytic mechanisms (1, 27) indicating the critical role of plasminogen activator in thrombolysis.

The significance of the present observations lies in the provision of direct experimental data in a region in which the evidence has, for technical reasons, been largely inferential. Hitherto, low levels of thrombolytic activity could only be assayed by indirect techniques that disturbed the balance of plasminogen system components in plasma and consequently the interpretation of such assays was uncertain (3). The present method for assay of thrombolytic activity is direct, employs unaltered plasma, is sufficiently sensitive for detection of low levels of activity, and the interpretation of results is unambiguous. Consequently, the finding that plasma of normal individuals possesses demonstrable thrombolytic activity under these test conditions would seem to be of considerable significance in the elucidation of *in vivo* thrombolytic phenomena. In the past it has been suggested that the plasminogen-plasmin system may be in a state of dynamic activity (28, 29) and the present evidence, obtained with acceptable technical procedures, provides strong support for this contention.

The demonstration that increased plasma activator concentrations may exist without detectable change in plasma fibrinogen, plasminogen or antiplasmin (6) suggests either that plasma contains an inhibitor to plasminogen activator or that plasma activator, because of physicochemical conditions, acts preferentially to activate thrombus plasminogen rather than plasma plasminogen. The evidence is, however, no more than suggestive and work on this problem has been scanty (3).

It is of incidental interest that levels of plasma thrombolytic activity may be increased several hundredfold over those found in health by the intravenous infusion of purified plasminogen activator (27), a procedure used for therapeutic purposes (30).

SUMMARY

A sensitive measure of plasma thrombolytic activity has been described which involves determination of the release of radioactivity from isotopically-labeled human plasma clots immersed in unaltered plasma.

Using the isotopic clot assay, thrombolytic activity was determined in plasma from healthy adults, from adults following stress and following the administration of drugs, and from individuals with disease.

The results indicate that plasma from normal adults contains a plasminogen activator capable of lysing human plasma clots under conditions similar to those *in vivo* and that the quantity of this material varies in response to stress and drug administration and in disease.

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