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THE ASSAY OF THE HUMAN

FIBRINOLYTIC SYSTEM

DISSERTATION

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By

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I. INTRODUCTION

Because of the increasing awareness that thrombosis causes more deaths in the United States than any other human disorder, the last decade has seen a tremendous upsurge in scientific interest in the human thrombolytic system. Many investigations have been undertaken to study the phenomenon of clot lysis in the test tube and in experimental animals. The primary goal of this research has been to understand the biochemical basis of clot lysis and, equipped with this knowledge, to discover agents to induce the thrombolytic state in prophylactic and therapeutic treatment of the disease states involving the presence of intravascular clots.

Fibrin, the insoluble protein which forms the matrix of the clot, is derived from fibrinogen by an enzymatic transformation which involves thrombin and ionic calcium. The clot is dissolved by a fibrin-attacking enzyme, plasmin, which degrades fibrin into several soluble proteolytic products. Plasmin, not usually present in large amounts intravascularly, is present in its zymogen form, plasminogen, which can be transformed to plasmin by several activators.

The agents causing plasminogen activation may be arbitrarily categorized into two major divisions; namely, the physiological and the nonphysiological activators. The physiological activators are those which are either inherent in the human system or which require the

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In order to study the extent of the clot-lysing capability of blood, either naturally or artificially induced, the development of reliable assays is necessary. Since the thrombolytic system involves a double activation, specific assays for each stage are necessary to elicit more information on the mechanism of these activations. Because it is extremely difficult, if not impossible, to separate plasminogen from proactivator in purification procedures currently used, individual assays of the active forms of these components becomes a challenging technical problem.

Since plasmin is a proteolytic enzyme, available methods for its assay include several protein substrates. When a physiological clot is the substrate, thrombolytic activity of plasmin is measured. When the substrate is a clot formed artificially by mixture of purified thrombin and fibrinogen, the fibrinolytic activity of the enzyme is obtained. When casein, a protein susceptible to digestion by plasmin is the substrate, the caseinolytic activity of plasmin is measured. The ability of most proteolytic enzymes, including plasmin, to hydrolyze certain synthetic esters has been exploited to develop assays using synthetic esters as substrates. In these procedures the esterolytic activity of plasmin (and perhaps activator) is measured.

The basis of most assays currently in use depends on the capability of plasmin (1) to dissolve fibrin clots (fibrinolysis), (2) to digest casein (caseinolysis), and (3) to hydrolyze synthetic esters (esterolysis).

presence of an entity usually present in the human system though they themselves are foreign substances. Thus, physiological activators can be placed in either of two subdivisions: (1) the direct physiological activators, urokinase and spontaneous blood activator (the latter present in the human plasma), which convert plasminogen to plasmin directly, and (2) the indirect physiological activators not normally present in the human plasma but when present cause a direct physiological activator to be formed. Streptokinase and staphylokinase are examples of this sub-category. Their action is to transform a pro-activator into a plasminogen activator.

The nonphysiological activators, on the other hand, are those entities which are foreign to the human organism and which, without the requirement of human proactivator, promote the fibrinolytic state either by transforming plasminogen to plasmin or, in some manner, increase the effectiveness of plasmin's ability to digest fibrin.

It is emphasized that, as in the formation of plasmin, activator is usually present in an inactive form called proactivator. Thus, a double activation is necessary to bring about a thrombolytic response. Proactivator must be changed to activator followed by plasminogen being transformed to plasmin.

The natural activation of the intravascular thrombolytic system remains a mystery although it is known that after severe exercise, shock or other body stresses, the clot-lysing properties of plasma or serum are significantly increased. The injection of large amounts of streptokinase usually have a similar effect.

II. SURVEY OF THE LITERATURE

A. The General Mechanism of Fibrinolysis

The current concept of the phases of activation and inhibition of the human fibrinolytic system is presented in Figure 1 (1). The chart illustrates that activation of plasmin from its precursor, plasminogen, can be accomplished either directly or indirectly. The indirect activation depends upon the activation of a proactivator before plasminogen can be converted to plasmin. In regard to these two methods of activation, new terms were introduced to differentiate the two mechanisms (2). Lysokinases are those activating agents which react with a proactivator to form the activator which transforms plasminogen to plasmin. Plasminoplastins are those entities that direct the plasminogen to plasmin transformation without the intermediate dependence of proactivator.

The interaction of the clotting and thrombolytic mechanisms has recently been made apparent. Generalized acceleration of coagulation upon addition of streptokinase and its enhancement by incubation of the treated blood with heparin has been observed (3). Another study noted initial hypercoagulability upon addition of streptokinase or urokinase to in vivo systems (4). An in vivo study demonstrated definite correlation between proteolysis, fibrinolysis, and coagulation. Induced intravascular coagulation followed by injection of

A review of pertinent published information is presented in the next chapter to provide the reader with a background of the mechanism and properties of the components of the human fibrinolytic system. In the review, there is more detailed discussion of the three categories of assays, briefly mentioned above, which are currently being used by investigators. Some of the more serious problems confronted when utilizing these assays are emphasized with the intent of making the reader aware of the serious need of a new assay system which circumvents these difficulties.

The purpose of this study initially was to develop an assay system with fibrin as the substrate specific for the individual components of the human fibrinolytic system. Requirements of the assay were that it be simple, reproducible and easily adapted for use in the clinical laboratory. Other objectives were to study the adsorption characteristics and the activation kinetics of the components of the fibrinolytic system.

INDIRECT ACTIVATION

Lysokinase
streptokinase
staphylokinase
tissue kinase
chemicals (?)

Proactivator → Activator

INDIRECT INHIBITION

{ ε-aminocaproic acid
kallikrein

DIRECT ACTIVATION

Tissue Activator
Urokinase
Technical Activation

Plasminogen → Plasmin

DIRECT INHIBITION

{ antitrypsin
antiplasmin

Fibrin (and other proteins) → Proteolytic Products

Fig. 1. Activation and Inhibition of the Human Fibrinolytic System (1)

FIGURE 1

Activation and Inhibition of the Human Fibrinolytic System (1)

plasminogen from rabbit, dog, cat and monkey and totally ineffective with bovine or porcine plasminogen or plasma (13).

A lysokinase of much lesser importance than streptokinase is staphylokinase, a product of certain strains of staphylococci (14, 15). It reacts more slowly than streptokinase in the human but activates plasminogen of various animals more effectively than streptokinase. Bovine plasminogen is not activated by staphylokinase (16).

2. Direct physiological activation. At the present time it is not known whether the spontaneous plasminogen activator in blood after electroshock, pyrogen injection, and severe exercise (17) is the same as that formed after treatment with streptokinase. It is believed that this "natural" activator is formed by the action of a tissue lysokinase on proactivator (18).

Tissues from various sources contain plasminogen activator or plasminoplastin activity (19,20). Body fluids from a hydrocoele, hydarthron and hygroma as well as amniotic fluid and saliva have plasminogen activator activity (21). The acid polysacharides in these fluids may be the plasminoplastins which in some unknown manner activate plasminogen (22). Activation of plasminogen in cartilage with a subsequent attack on the protein-chondroitin sulfate complex has been reported (23).

It has been suggested that blood trypsin may be the activator of the plasminogen to plasmin as well as the prothrombin to thrombin

streptokinase produces a pronounced physiological effect which involved a drastic drop of blood fibrinogen. Neither induced coagulation nor did streptokinase injection alone have this effect (5).

B. Activation of Plasminogen

1. Indirect physiological activation. Early studies have demonstrated that a bacterial protein produced by growing hemolytic streptococci (6) activates the lytic precursor, plasminogen, to plasmin (7). This bacterial product, streptokinase, has been more extensively used as a plasminogen activator in currently reported experiments than any other activator. Streptokinase exhibits little activity in any species other than the human. It first reacts with a precursor in human plasma to form a plasminogen activator which causes the transformation of plasminogen to plasmin in many species (8,9,10). The stoichiometric and nonenzymatic potentiation of the precursor, pro-activator, was emphasized in a later study (11). Though the enzymic nature of streptokinase remains in doubt, esterase activity with 2-naphthyl acetate as the substrate has been demonstrated. This esterase activity is not inhibited with soybean trypsin inhibitor (12).

Since the role of streptokinase is an indirect one in that it triggers an activation of plasminogen by combining with an inherent component of human blood, it has been listed as an indirect physiological activator or lysokinase. It is important to note that though streptokinase acts on human plasminogen, it is much less effective on

activation is associated with the release of about sixteen percent of the nitrogen of the plasminogen molecule (26,29).

3. Nonphysiological activation. Of the organic solvents known to activate plasminogen, only chloroform has been studied in any detail. When plasma, serum or euglobulin derivative thereof is mixed with small but specific amounts of chloroform, the plasminogen in these materials is slowly transformed to plasmin (33,34). In an earlier study the possibility was raised that chloroform merely destroyed the inhibitors of plasmin and without their interference autocatalysis by plasmin takes place (35). Another theory has chloroform releasing an activator from its inhibitor (15,36). If the true mechanism of chloroform activation follows the inhibitor destruction theories presented above, then chloroform would participate as an indirect physiological activator since the activation of plasminogen is ultimately accomplished by a released physiological activator (which would be plasmin in the case of autoactivation). Since the information on chloroform activation is still quite limited, it is arbitrarily classified as a nonphysiological activator.

Though trypsin was discussed previously as a direct physiological activator, it may also be a nonphysiological activator (24). In the absence of inhibitors and in the presence of stabilizing agents such as glycerol, the activation kinetics for pancreatic trypsin are those of a first order enzymatic reaction. The activation is slower than that induced by streptokinase or urokinase (26).

systems. It was further proposed that both proenzymes may arise from the same precursor protein (24).

It has been noted that plasminogen prepared from plasma or serum is spontaneously activated under certain conditions (25). The auto-activation takes place slowly in a medium containing a large percentage of glycerol and is accomplished without the participation of plasminoplastins (26).

The plasminoplastin of prime interest in current investigations is urokinase or urine activator discovered in urine of man and other animals over a decade ago (27). This activator with reported weak proteolytic activity (28) has been concentrated and partially purified by chromatography on a silicic acid column with ammonium sulfate precipitation of the eluate (29), by adsorption on silica gel followed by aqueous ammonium hydroxide elution with further purification on ion exchange columns (28), and by barium sulfate adsorption with alcohol precipitation of the eluate (30). In the latter purification technique it was demonstrated that contaminating uropepsin was destroyed at the alkaline pH used in one of the purification steps. Unlike the relative specificity demonstrated by streptokinase for the human system, urokinase from one species can activate in varying degrees plasminogen from another species (31). Urokinase has esterolytic activity against tosyl arginine methyl ester and lysine ethyl ester (32). The conversion of plasminogen to plasmin by urokinase is an enzymatic process which proceeds with first order kinetics. The

The euglobulin precipitation technique commonly used at present is essentially a dilution of human plasma with distilled water followed by acidification to pH 5.2 or 5.3 with precipitation occurring at about 0°C. (45,30). The euglobulin precipitate reportedly contains the total plasminogen content of the original plasma and is essentially free of any inhibitors (30). The lack of inhibitors in the euglobulin fractions has been emphasized (46,47). The use of euglobulins as a source of plasminogen considerably reduces the interference of inhibitors in plasmin determinations. It is well known that the euglobulin preparations contain a large amount of proactivator in addition to plasminogen. Proactivator activity in euglobulins has been used as an index of total proactivator in plasma (48).

Cohn's (49) human plasma fraction III is currently used as the primary starting material for further purifications of plasminogen after it was reported that this fraction contained virtually all of the plasminogen in plasma (50). Further fractionation of fraction III, accomplished by variation of temperature, ionic strength and alcohol concentration, enabled the separation of subfractions III-1, III-2 and III-3. The last of these subfractions was demonstrated to contain most of the plasminogen. No other biologically active entity could be demonstrated in the subfraction. Methods for the further purification of plasminogen were initially developed utilizing the relative stability of plasminogen at low pH. The most widely used of these acid extraction procedures is the one developed by Kline (51).

The mechanism of nonphysiological activation is not understood because there are so many varied and unrelated chemical agents that enhance fibrinolytic activity. Since the chemicals having this activating property have no apparent chemical similarity, there is no known structural basis to explain the mechanism of their activating ability. The knowledge of these enhancing agents dates back to the turn of the century when thiourea was shown to promote clot lysis (37). Many other compounds including naphthalene sulfonate and naphthoates (38), 2,4-dimethyl sulfonate (39), fatty acids (40), and peptone (41) were observed to have this property. A relationship between structure and so-called fibrinolysis "induction" has been described in a study of a large variety of aromatic carboxylic and sulfonic acids (42). An interesting observation was that all the aromatics having a fibrinolytic effect were hydrotropic. With the sulfonated aromatics caseinolytic and fibrinolytic activity increased as the number of methyl groups on the benzene ring increased. Enhancement of streptokinase-activated esterase activity of purified plasminogen in the presence of phosphatidyl inositol, phosphatidyl serine, asolectin, and cephalin in the incubation mixtures has been reported. While esterase activity was increased, caseinolytic activity was not affected (43).

C. Source and Purification of Plasminogen

Agglutinins precipitated from human plasma and redissolved were first used as a partially purified source of human plasminogen (44).

DEAE Sephadex has been used recently in purifying plasminogen. Lysine solutions have been utilized as eluting solvents with DEAE Sephadex columns (56, 57) as well as with those of DEAE cellulose (58). Lysine and ϵ -aminocaproic acid appear to have a specific solubilizing effect on plasminogen. Paper electrophoretic analyses of DEAE Sephadex eluates indicated that the plasminogen was apparently concentrated in a minor protein band. A Sephadex gel filtrate of plasma fraction II-III was further purified on a DEAE Sephadex column (59).

None of the elaborate purification techniques discussed above have resulted in a plasminogen preparation devoid of activator activity upon addition of streptokinase. Indeed, the purest form of plasminogen obtainable from human blood contains the so-called proactivator which is converted in the presence of streptokinase into a plasminogen activator (60). Because the two entities, plasminogen and proactivator, have not been separated, some investigators believe they are identical (61, 62, 63). In a recent study, however, Greig and Cornelius reported that a separation of plasminogen-rich and proactivator-poor solution from one with the opposite characteristics was accomplished (64). These investigators believe that, though proactivator and plasminogen differ, they are derived from the same parent molecule. The existence of the unique entity, proactivator, remains in doubt.

D. The Enzymatic Properties of Plasmin

Plasmin is formed from plasminogen by the action of one of many activators. Plasmin has a molecular weight of about 107,000,

In this method fraction III is extracted at pH 2; then the extracted material is reprecipitated at pH 5.3 and redissolved at pH 11. The plasminogen was again precipitated at pH 5.3 and dialyzed against a sodium phosphate solution. The final product was insoluble at neutral pH. More recently two more steps, those of 0.1 M lysine extraction and precipitation at pH 2 in 1.0 M sodium chloride, were added to the basic procedure to give even greater purity of the proenzyme (52). Acid extraction, however, may denature plasminogen (17). "Native" plasminogen had peak caseinolytic activity at an enzyme-substrate ratio of 1:40 while the plasminogen obtained by the above described acid extraction method had peak activity at 1:200 to 1:300 (53). In view of the above-mentioned differences in activity and because the acid extracted plasminogen is insoluble while plasminogen purified by other means is soluble, the acid extracted entity is referred to as Kline's plasminogen to distinguish it from the so-called "native" variety.

Chromatography has been extensively used in obtaining highly purified preparations of plasminogen. Extraction of plasma with 30 percent ammonium sulfate followed by precipitation at pH 5.3 and chromatography of the redissolved precipitate resulted in a purified water-soluble preparation of plasminogen (54). The utilization of DEAE cellulose columns on ϵ -aminocaproic acid extracted euglobulin fractions as starting material resulted in highly purified preparations of plasminogen (55). In at least two electrophoretically different peaks plasminogen was demonstrated.

E. Inhibitors of the Human Fibrinolytic System

Figure 1 illustrates two categories of inhibitors in the human fibrinolytic system. The antiplasmins are those agents that directly inhibit the action of plasmin on its substrate, while the antiactivators have an effect on the activator and cause a suppression in the conversion of plasminogen to plasmin.

The antiplasmin of blood has received special attention from investigators since the possibility exists that it may be directly involved in pathologic conditions involving clot dissolving abnormalities. The possibility that the known blood trypsin inhibitor and antiplasmin are the same entity has been obviated in a blood fractionation study which demonstrated that trypsin inhibitor was in fraction IV while plasmin inhibitor, almost completely free of trypsin inhibitor was in fraction V (36). Verification of this observation was obtained in ammonium sulfate fractionation studies (71) as well as in electrophoretic experiments. Antiplasmin activity was present in the α_2 globulin fraction, whereas the major antitryptic activity was in the α_1 globulin fraction.

More recent studies have demonstrated the presence of more than one antiplasmin in the blood. In one of the more interesting of these studies the investigators isolated two antiplasmins which they called α_1 and α_2 due to their electrophoretic migration characteristics (73). α_1 inhibitor reacted immediately with plasmin but did

approximately twenty-four percent less than that of its zymogen. In the conversion, a percentage increase in hexose content indicates that this moiety was not split off during activation (65).

The proteolytic activity of plasmin has been extensively studied. It has been concluded that plasmin catalyzes the cleavage of the same synthetic esters as does trypsin and thrombin (10,66). Plasmin and trypsin have similar kinetic behavior with similar substrates, but both differ considerably from thrombin. It was proposed that five active centers are present in both plasmin and trypsin from kinetic data obtained in pH profile studies (67). Probably one or more of these are different in the two enzymes. One of the differences noted between plasmin and trypsin was their storage stability.

Since trypsin and plasmin are similar, it was concluded that plasmin cleaves an arginyl-glycyl bond when it attacks fibrin (68). The kinetic properties of human plasmin are similar to those of bovine plasmin (69).

The presence of more than one type of plasmin has been suggested by investigators studying the kinetic behavior of streptokinase-activated plasminogen preparations (70). It was suggested that three plasmins are normally present, one with a fast rate of proteolytic activity, the second having a slower rate of proteolysis, and the third with primarily esterolytic activity.

attributed to their high phospholipid content (83). This conclusion is somewhat contradictory to the observation that certain phospholipids have an enhancing effect on the system (43).

In addition to the antiplasmins, another heat-labile factor present in human serum and plasma makes clots more resistant to plasmin attack. The factor is not an antiplasmin and its effect may be due to substrate alteration (fibrin) and not to action on the enzyme system (84).

Known inhibitors of trypsin obtained from plant and animal sources also inhibit plasmin. Pancreatic trypsin inhibitor (85) as well as soybean trypsin inhibitor (86) are very effective inhibitors of the plasmin. The ability of soybean trypsin inhibitor to inhibit plasmin and not activator has been reported (87). A method to differentiate between the esterolytic activity of plasmin and that of activator by suppressing the former with the inhibitor is proposed.

Very little information is available on the antiactivators or the inhibitors of plasminogen activation. Inconclusive evidence suggestive of the presence of a natural antiactivator in circulating blood has been presented (15). A bovine globulin preparation which inhibits activation of bovine plasminogen by human globulin treated with streptokinase was reported (88). The effect of bovine globulin on the activation process was markedly reduced in the presence of fibrin. This is another demonstration of the protection by fibrin of the components of the fibrinolytic system from their inhibitors.

not combine with the enzyme in a fixed ratio. The addition of more protein substrate to the reaction mixture reversed the inhibition. Thus, α_1 antiplasmin acts as a competitive inhibitor. The α_2 antiplasmin, on the other hand, reacted slowly and in a fixed ratio with plasmin. The addition of more protein substrate did not reverse the inhibition but prevented further inhibition. With in vivo systems, the presence of increased amounts of the natural substrate, fibrin, therefore, may reverse or suppress fibrinolytic inhibition. Addition of antiplasmin to streptokinase, urokinase, or spontaneously activated human plasminogen resulted in a more rapid decrease of the caseinolytic activity than the in vivo fibrinolytic activity (74). Antiplasmin, therefore, is a less effective competitive inhibitor of the plasmin-fibrin system than of the plasmin-casein system. More studies are necessary to fully understand the competitive nature of the antiplasmins.

Synthetic ester substrates for plasmin are competitive inhibitors of plasmin's caseinolytic activity. The esters, p-toluenesulfonyl L-arginine methyl ester and L-lysine ethyl ester, compete with casein for plasmin (11,75).

The many known irreversible inhibitors of plasmin include cysteine and mercaptoethanol (76), heparin (77), carboxylic and amino acids (78), polyamino acids (79), methylamine and urea (80). Serotonin (81) and glycerol (82) are inhibitors of fibrinolytic activity. The ability of blood platelet preparations to inhibit fibrinolysis was

for the release of three fibrinopeptides from fibrinogen instead of the two previously mentioned (98). Fibrinogen loses about eighteen percent of its sialic acid during clotting. If this sialic acid is removed by neuraminidase (instead of by thrombin), the fibrinogen becomes much more susceptible to clotting (99). Kinetic studies demonstrated that above pH 8, two first order reactions occurred in the thrombin-catalyzed conversion of fibrinogen to fibrin (100). At pH 5.5 to 8 only one reaction was apparent. The interpretation of the two reactions noted at higher pH was that the first reaction was proteolysis wherein fibrin molecules were formed, while the second reaction was the polymerization reaction.

A second stage of thrombin action is the activation of an oligosaccharide-splitting enzyme which, in its activated form, "vulcanizes" the clot and makes it insoluble in 5 M urea. Clots lacking this factor are soluble in the urea solution (101). The clot's resistance to urea suggests that the so-called vulcanization makes the fibrin clot less susceptible to depolymerization.

A two-stage role of thrombin has also been proposed in another study. Thrombin forms fibrin from fibrinogen and "desmofibrin" from fibrin by the activation of a fibrin-stabilizing factor analogous to the oligosaccharide-attacking enzyme (102). An enzyme called "fibrinase" which changes urea-soluble clots to urea-insoluble clots has been isolated and purified (103).

Fibrinolytic activity is reduced by ϵ -aminocaproic acid in vivo as well as in vitro. The compound corrects in vivo defects which result from pathological increases of fibrinolytic activity (89). Tube clot lysis studies indicate strong inhibition of lysis by the caproic acid (90). The mechanism by which the compound suppresses fibrinolytic activity is primarily inhibition of the activator, i.e., the conversion of plasminogen to plasmin (91,92). Though the antiactivator effect is considered the primary influence, evidence for antiplasmin activity of ϵ -aminocaproic acid has been reported (93).

An investigation involving the serum of a patient with extended recurrent intravascular clotting established that in addition to the normal antistreptokinase antibodies contained in fraction III-II, a heat-labile antiurokinase was present in fraction IV-I (94). More recently, a normal component in human serum was reported to inhibit the activation of plasminogen by urokinase (95).

F. The Formation and Lysis of Fibrin

Because fibrin is the important physiological substrate for the fibrinolytic system, its origin, properties and degradations are worthy of discussion.

The action of thrombin on fibrinogen was reported to cause the loss of three percent of the fibrinogen molecule as two peptides (96). These altered fibrinogen molecules, now fibrin molecules, aggregate to form the primary clot (97). A more recent study reported evidence

Plasmin hydrolysis of fibrin results in twelve non-dialyzable products. The concentration of trichloroacetic acid-soluble products progressively increased with continued incubation of the reaction mixture (109). These products were separated and identified using electrophoretic procedures.

It is of interest to note that some studies of clot lysis involve fibrin clots containing plasminogen incorporated within the clot itself. In one of these studies, the amount of solubilized protein released from a clot exposed to an activator solution was proportional to the amount of plasminogen within the clot. If plasminogen is added to the aqueous phase, no increase in activity occurs. With a constant amount of plasminogen incorporated in the gel phase, the rate of fibrinolysis was a measure of the amount of activator (urokinase or streptokinase-induced plasma activator) added to the system (110). In another study plasminogen adsorbed in the forming clot had little effect on the lysis. Adsorption of activator and plasminogen on the surface of the formed clot caused formation of plasmin with subsequent lysis (111).

G. Measurement of Human Fibrinolytic Activity

1. General. The assays for the components of the human fibrinolytic system may be classified according to the substrate used. Some assays not currently used and of relatively little importance will be omitted from this discussion.

It has been demonstrated that even the purest preparations of fibrin or its precursor, fibrinogen, are contaminated with plasminogen (104,105,106). The importance of endogenous plasminogen in fibrin preparations used as substrates in assays of the components of the fibrinolytic system has been emphasized earlier (9,17). It has recently been concluded that bovine fibrin contaminated with plasminogen is useless for the estimation of plasmin in solutions containing plasminogen activators. Fibrinolytic activity measured on systems from which the contaminating plasminogen has been removed is true plasmin activity. Bergstrom and Wallen have developed a method for fibrinogen purification in which such removal of endogenous plasminogen was accomplished by extraction of the contaminant with a lysine solution (107). Later it was demonstrated that ϵ -aminocaproic acid as well as lysine solutions change the solubility characteristics of the plasminogen-contaminated human fibrinogen solutions. This solubility effect was utilized to prepare fibrinogen free of the proenzyme (108). It was observed in this study that dilution of a solution of human fibrinogen in 0.3 M sodium chloride with lysine solution at pH 7 to a final lysine concentration of 0.1 M and precipitation at an ethanol concentration of seven percent results in a fibrinogen preparation in 95 percent yield containing less than one-tenth of the original plasminogen. When the purified fibrinogen was converted to fibrin and treated with plasmin, substrate reactivity was unchanged. No apparent changes in the electrophoretic or sedimentation characteristics of the lysine-extracted fibrinogen were noted (108).

obtained either by the addition of an exogenous activator such as urokinase or by formation of intrinsic activator by the addition of streptokinase to a test solution exposed to a plasmin-specific substrate. For evaluating individuals for intravascular fibrinolytic activity, the plasma euglobulin has been universally accepted as the enzyme source that is free of inhibitor suppression effects. To evaluate enzyme activity in the presence of its natural inhibitors, plasma has been predominantly used as the enzyme source.

2. Casein as the substrate. Casein has been widely accepted as a substrate for plasmin assay. In the original method, the extent of enzymatic digestion of casein was measured by the amount of acid soluble proteolytic products detected spectrophotometrically (at 280 mu) in trichloroacetic acid extracts of the incubation mixture (112). In another method developed about the same time a lesser concentration of casein was utilized in the incubation mixture with aqueous perchloric acid as the extracting medium instead of trichloroacetic acid (113). Since the publication of the above caseinolytic methods, several modifications have been suggested (114,115). A method has been proposed in which the radioactivity of acid-soluble proteolytic products of I^{131} labeled casein was used as a measure of plasmin activity (116).

A recent study has given evidence that the α_1 and beta fractions of casein are less sensitive to plasmin attack than the α_2 fraction (117). Because purified casein contains these three fractions

It should be evident that no matter what substrate is utilized, the activity being measured may not be that of plasmin alone, but a combined effect of plasmin and activator. It is important, therefore, to consider not only the method involved but also the entity being measured.

The measurement of the fibrinolytic precursors, plasminogen and proactivator, can obviously be done only after these substances have been transformed into their active forms. A plasminoplastin such as urokinase transforms all plasminogen to plasmin. The difference in activity with and without urokinase treatment gives a plasminogen value while the activity without urokinase treatment gives the plasmin value. Since urokinase may have an enzymatic effect itself on the substrate, knowledge of urokinase's ability to hydrolyze the substrate is very important in evaluating activity in such experiments. Streptokinase completely changes proactivator to activator and subsequently all plasminogen is transformed to plasmin. Since all the plasminogen preparations are mixtures of proactivator and plasminogen, the addition of streptokinase enables the measurement of total activator (proactivator plus activator) and total plasmin (plasminogen plus plasmin) activity if substrates can be made to respond specifically with either plasmin or activator.

Thus, total activator activity can be obtained by the presence of optimal amounts of streptokinase in reaction mixtures containing a specific substrate for activator. Total plasmin activity may be

only LME (11). No corroborating evidence has been reported. A later study demonstrated two distinct LME hydrolytic enzymes activated in euglobulins by streptokinase (121). These enzymes may be differentiated since one of these enzymes was relatively heat-stable, inhibited by soybean trypsin inhibitor and not inactivated by precipitation at pH 2 in 2 M sodium chloride solution, while the other had none of these characteristics. In another investigation an assay in which LME was utilized as substrate was proposed for activator activity. The esterase activity of activator, formed in the presence of streptokinase, was not inhibited by soybean trypsin inhibitor, while plasmin esterase activity was completely suppressed by the presence of the inhibitor in the reaction mixture (87). The ability of the activator to hydrolyze esters is not universally accepted since it has been recently reported that streptokinase-activated esterase activity is independent of the activator titer and depends only on the fibrinolytic titer of plasmin itself (122).

Aside from the confusion about the fibrinolytic component giving esterase activity in activated plasminogen preparations, the ability of other enzymes such as trypsin (66), thrombin (123), and thrombo-kinase (124) to hydrolyze the above-mentioned esters suggests that the esterolytic assays be limited to well-purified sources of plasminogen since the presence of these other proteolytic enzymes in the reaction mixtures will confuse results.

in varying amounts, it was concluded that up to fifteen percent variation in results have been observed with various casein preparations used in the assay.

Although casein digestion was thought to be exclusively due to plasmin activity in assays of the human fibrinolytic system, it has recently been reported that the activator formed by the presence of streptokinase also has caseinolytic activity (87). If this observation is correct, then caseinolytic methods measure the cumulative activity of plasmin and the activator's proteolytic activity in all the assays previously thought to measure plasmin activity alone. More information on the properties of the activator formed by the addition of streptokinase is required before definite conclusions can be made concerning the casein assays.

3. Synthetic esters as substrates. The esters most widely used to measure the esterase properties of the fibrinolytic components are the methyl ester of lysine (LME) (118) and the methyl ester of p-toluene sulfonyl arginine (TAME) (119). The extent to which these esters have been hydrolyzed after the incubation period of the assay has been variously measured; i.e., the titration of the released acid (60), spectrophotometric measurement of a derivative of the released acid (120), and the spectrophotometric measurement of the hydroxamic acid derivative of the non-hydrolyzed ester (119).

It has been reported that plasmin hydrolyzes both LME and TAME, while activator (formed in the presence of streptokinase) hydrolyzes

index of fibrinolytic activity of the test solution. In a modification of the fibrin clot procedure, layers of fibrin are formed in petri dishes resulting in "fibrin plates" (47,129). The volume of fibrin lysed by a test solution placed on its surface in a twenty-four to forty-eight hour incubation period is the measure of the solution's fibrinolytic activity. Heating bovine fibrin plates at 80°C. for fifteen minutes rendered all endogenous plasminogen inactive. The plasmin activity only would be measured in any solution tested with these heated plates since the activator in these solutions would have no usable plasminogen to convert to plasmin (130). This observation was supported by another study in which heating human fibrin to 80°C. for ten minutes destroyed all the endogenous plasminogen. Thirty minutes at this temperature, however, had no effect on endogenous proactivator (128). A limitation in the utilization of heated fibrin plates in plasmin assays was pointed out by the demonstration of reduced sensitivity to lysis by plasmin on heated fibrin plates as compared to unheated fibrin plates (60). This observation implies that a physical change occurred in the heated fibrin altering its substrate characteristics.

Fibrin clots prepared from I¹³¹-labeled fibrinogen are the substrates in recently described clot lysis assays (60,131). The radioactivity released into the aqueous phase is a measure of fibrinolytic activity of the test solutions. Assays based on the inherent fluorescence of fibrinolytic products from fibrin clots (132) as well

In conclusion it is interesting to note that in a test of several plasminogen preparations the ratios of casein to ester hydrolytic activity were not constant (125). The same lack of correlation was observed when fibrinolytic and esterolytic activities of streptokinase-treated plasminogen preparations were compared (126).

4. Fibrin as the substrate. The majority of the methods in which fibrin is the substrate are based on preformed clot dissolution as a measure of plasmin activity. Thrombin-induced clots of whole plasma have been used for the estimation of total fibrinolytic activity of test solutions (42,127). The volume of clot dissolved after a given incubation period with the test solution is the measure of fibrinolytic activity. It is generally agreed that the lytic activity of the test solution is due to its activator activity which transforms the plasminogen within the clot to plasmin which in turn causes lysis. An advantage of using the plasma clot is that it is a measure of over-all fibrinolytic activity in the presence of natural inhibitors.

Fibrin clots have also been used with satisfactory results (54, 128). The fibrinolytic activity of test solutions using fibrin clots as substrate was due primarily to the activator in these solutions (with plasmin formed from the endogenous plasminogen in the fibrin clots). In all the fibrin methods either the volume of clot dissolved in a given time or the time required for complete dissolution was the

also easily prepared from commercially available salts of these amino acid esters and are relatively very stable. The primary advantage of using fibrin as the substrate in assays of the human fibrinolytic system is readily apparent since fibrin is the natural physiological substrate for plasmin. This advantage seems over-riding to those discussed above for those assays using a substrate other than fibrin since many valid analogies can be made from in vitro laboratory measurements of fibrinolytic activity to this activity present in the human intravascular system.

From the above discussion concerning the individual characteristics of the available assays of human fibrinolytic activity, it is concluded that a vital need still exists for a simple, specific and reproducible assay of plasmin and activator activity. An ideal assay system would be one in which all of the undesirable features of the previously available assays were eliminated and in which many of the desirable aspects of these assays have been incorporated.

as the fluorescence released from fluorescein-treated fibrin clots (133) have also been reported. The release of hemoglobin from clots prepared in hemoglobin solutions is also an index of fibrinolytic activity (90).

It is generally accepted that the amount of fibrinolysis of pre-formed fibrin clots is directly related to the clot surface; it is, therefore, essential to keep clots as uniform as possible. With clot lysis procedures it is concluded that the extent of fibrinolysis is not only dependent upon plasmin or activator concentrations, but also upon the surface area of the clot exposed to the test solution.

The general types of assays employed in studying the human fibrinolytic system have been classified and discussed in the preceding paragraphs according to the substrate used. Each substrate, as previously mentioned, has individual properties which tend to limit the applicability and accuracy of the assay system in which it is utilized. Not mentioned, however, were the advantages that assay systems using a particular substrate may have over assays in which another substrate is used. The caseinolytic procedures have as their primary advantage a simple and convenient method to completely stop the proteolytic reaction and to estimate the extent of substrate hydrolysis by spectrophotometric measurement of acid-soluble proteolytic products. In the assays in which synthetic esters are the substrates the main advantage is substrate homogeneity. The stock solutions of any of the esters susceptible to plasmin activity are

Laboratories, Pearl River, New York). These solutions were stored frozen (maximum storage time of three weeks) until just before use.

2. Purified urine activator (urokinase). Urine samples less than four hours old (supplied by the Clinical Laboratory, Department of Pathology, University Hospital) were combined and a total of 1500 ml was used as the starting material. The following modification of the Sgouris purification method (30) was used.

The urine was adjusted to pH 8 with 1 N sodium hydroxide and cooled to 0° C. After three hours at this temperature the supernatant was decanted and the precipitate discarded. The pH of the supernatant was then adjusted to 4.5 with 1 N hydrochloric acid. The solution was stored at 5° C. overnight, after which the clear supernatant was removed by aspiration and discarded and the precipitate was recovered by centrifugation at 0° C. and 2,000 X g for one hour. The precipitate was then dissolved in 75 ml distilled water. The solution was made alkaline (pH 8.5 ± 0.1) with 1 N sodium hydroxide and mixed with 1.5 grams of freshly prepared barium sulfate (obtained by reprecipitation of barium sulfate by dilution of a concentrated sulphuric acid solution in which a saturating amount of barium sulfate was dissolved). The mixture was stirred for thirty minutes with a magnetic stirrer. The barium sulfate with adsorbed materials was recovered by centrifugation at 1500 X g and 0° C. for thirty minutes. The precipitate was eluted twice with 2 percent sodium citrate. The sodium citrate was then treated with 2 percent barium chloride with addition of 1.5 moles

III. EQUIPMENT AND REAGENTS

A. Equipment Utilized

A Dubnoff Metabolic Shaking Incubator set at 37° C. and equipped with an insert to hold nine 25 ml Erlenmeyer flasks was used to shake the reaction mixtures (100 times per minute) during the incubation period.

The spectrophotometric measurements were performed in standard 1.0 ml matched quartz cuvettes in a Beckman DU spectrophotometer equipped with an optical density converter, cuvette positioner and absorbance indicator (Models 200, 210 and 220 respectively, manufactured by the Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The manual cuvette selection procedure for measurement of absorbance of individual samples was used.

An International Refrigerated Centrifuge, Model PR-1, was used for all centrifugations.

All glassware was washed with laboratory detergent followed by three rinses with distilled water and dried in an oven at 150° C.

B. Source and Preparation of Activators

1. Streptokinase. A solution containing 4,000 Christensen units per ml phosphate buffer (Item 1 under Miscellaneous Reagents) was prepared from vials of streptokinase-streptodornase "Varidase" (Lederle

of barium chloride for each mole of sodium citrate added. The precipitated barium citrate was discarded after centrifugation at 0° C. and 1500 X g for thirty minutes. The clear supernatant was adjusted to pH 4.5 with 1 N hydrochloric acid and enough ethanol (95 percent) was added to obtain a 20 percent (v/v) alcoholic concentration. The temperature was maintained at 0° C. during and after ethanol addition. After thirty minutes at this temperature, the resulting cloudy solution was centrifuged at 1500 X g and 0° C. for one hour. The precipitate was redissolved in 30 ml phosphate buffer. Aliquots of the purified urine activator solution were put in separate vials and stored frozen until just before use (maximum storage time of six weeks). Each ml of the solution represented 50 ml of the initial starting material.

C. Preparation of Substrates

1. Bovine fibrin suspension (BFS). A known amount of purified, washed bovine fibrin (Mann Research Laboratories, New York, New York) was ground to a fine powder with a mortar and pestle and suspended in 1 percent Tween 20 phosphate buffer (Item 2 under Miscellaneous Reagents) using 20 ml for each gram of fibrin. After the course suspension was kept at 5° C. overnight, it was ground in a large Potter-Elvehjem type tissue grinder until it passed through the grinder without difficulty. The fine suspension was then transferred to a centrifuge bottle and centrifuged at 1500 X g and 5° C. for thirty

minutes. The sediment was thoroughly resuspended in 0.1 percent Tween 20 phosphate buffer (Item 3 under Miscellaneous Reagents) again using about 20 ml for each gram of fibrin.

The concentration of fibrin in the washed fibrin suspension was obtained gravimetrically. A 2.0 ml aliquot was filtered through a small, washed, air dried, and pre-weighed filter paper and washed with about 20 ml distilled water. The filter paper was air dried to a constant weight and the weight of the fibrin in the aliquot was found as the difference of the two weighings. The suspension was then adjusted to contain 3.0 grams of fibrin per 100 ml of suspension. It was important that suspensions be washed by centrifugation and resuspension in equal amounts of fresh 0.1 percent Tween 20 phosphate buffer at least twice before use since failure to do so occasionally led to high substrate blanks.

The rate at which the suspension settled varied somewhat with each preparation; however, no difficulty was encountered in obtaining accurate aliquots if each pipetting was preceded by thorough mixing of the suspension. The suspension was frozen and stored until just before use (maximum storage of six weeks). There was no apparent effect of the freezing and storage on activity when comparison was made with non-frozen freshly prepared suspensions.

2. Lysine extracted bovine fibrin suspensions (LEFS). In an attempt to remove endogenous bovine plasminogen from the bovine fibrin

of Physical Chemistry Related to Medicine and Public Health, Harvard University, Boston 15, Massachusetts) was prepared. The solution was stored frozen (maximum storage time of two weeks) until just before use.

2. Plasma. Sixteen whole blood mixtures (3 mg/ml potassium oxalate as anticoagulant), containing ten to fifteen combined individual specimens for each mixture, were prepared from specimens supplied by the Clinical Chemistry Laboratory, Department of Pathology, University Hospital. All individual specimens were processed less than three hours after the venipuncture. These whole blood mixtures were centrifuged at 1500 X g and 5° C. for thirty minutes. The plasma was removed from each centrifuged blood mixture and stored frozen until used (maximum storage time of two weeks).

3. Euglobulin. Sixteen plasma specimens obtained by the procedure described above, were processed to give euglobulin precipitates according to the method of Kowalski (45). The procedure is essentially the euglobulin precipitation after sixty minutes at 5° C. from a 1 to 10 dilution of plasma acidified with 10 percent acetic acid to pH 5.3.

After the euglobulin precipitates were recovered by centrifugation, they were dissolved with phosphate buffer to a total volume equivalent to the volume of plasma originally used in their preparation. The euglobulin solutions were stored frozen (maximum storage time of one week) until just before use.

suspension described above, a lysine extraction procedure, successful with human fibrinogen (107,108), was adapted for use with the fibrin preparation.

A known amount of the 3 percent bovine fibrin suspension (BFS) maximum of 6.0 ml was placed in a graduated 50 ml centrifuge tube and centrifuged at 2,000 X g and 5° C. for twenty minutes. To the residue was added 7.5 ml of 0.1 M lysine solution (Item 4 under Miscellaneous Reagents) for each ml of original fibrin suspension. The sediment was thoroughly resuspended maintaining the tube at 0° C. Frequent mixing of the suspension by tube inversion at the above temperature was performed for thirty minutes. Following centrifugation at 2,000 X g at 0° C. for twenty minutes, the resulting supernatant was discarded. The lysine extraction procedure was then repeated using a fresh amount of 0.1 M lysine.

The twice lysine-extracted fibrin, obtained as the sediment after the last centrifugation, was washed with 0.1 percent Tween 20 phosphate buffer by resuspension in the buffer and centrifugation. The washing procedure was repeated. The final sediment was then resuspended in a volume of 0.1 percent Tween 20 phosphate buffer necessary to bring the total volume of the suspension to its original value.

D. Preparation of Human Fibrinolytic Components

1. Plasma fraction III-3. A stock solution containing 1.0 mg/1 ml phosphate buffer of the fraction (from the University Laboratory

IV. STANDARD PROCEDURES

A. The Standard Assay

A series (maximum of nine) of acid washed 20 ml Erlenmeyer flasks containing 1.0 ml aliquots of the 3.0 percent bovine fibrin suspension (BFS or LEFS) were placed in the Dubnoff incubator (at 37° C.). After ten minutes of shaking 1.0 ml samples of test solutions* were added to flasks at one minute intervals. Included in this series were necessary blanks. Usually only a blank consisting of phosphate buffer as the test solution was required, except when testing concentrated plasma solutions in which case a blank of the plasma dilution used was also necessary. Additions were made without interruption of the shaking. The flasks were then stoppered and the shaking continued for exactly sixty minutes from the time that the first test solution was added. With the shaking maintained, 2.0 ml of 10 percent trichloroacetic acid was added at one minute intervals in the same order of addition as that of the test solutions so that an exact sixty minute interval was obtained for each flask. After the shaking was continued for another fifteen minutes, the flask contents were transferred to 15 ml centrifuge tubes. The tubes were kept at least two hours (preferably overnight) at 5° C. and then centrifuged at 2,000 X g and at 25° C.

*A test solution is defined as a solution to be assayed containing any or all of the following entities: proactivator, activator, plasminogen and plasmin.

E. Miscellaneous Reagents

1. Phosphate buffer. (0.1 M, pH 7.4) This buffer, prepared from the disodium and monopotassium salts by the University Laboratory Stores, was the only buffer used as diluent in the entire study.
2. 1 percent Tween 20 phosphate buffer. Tween 20 (Mann Research Laboratories, New York, New York) was added to phosphate buffer to give the concentration indicated (v/v).
3. 0.1 percent Tween 20 phosphate buffer. Tween 20 was added to phosphate buffer to give the concentration indicated.
4. 0.1 M lysine. An aqueous solution prepared from D,L-lysine hydrochloride and adjusted to pH 7.0, with 1 N NaOH.
5. 10 percent trichloroacetic acid. An aqueous solution prepared from the crystals of the acid (w/v).
6. Reagents for urine activator purification. 1 N sodium hydroxide, 1 N hydrochloric acid, barium sulfate, concentrated sulphuric acid, 2 percent sodium citrate (w/v), 2 percent barium chloride (w/v) and ethanol (95 percent).

2. Preactivated plasma fraction III-3 (PPF). The streptokinase preactivation procedure described above for plasma and euglobulin was also used for the higher concentrations of fraction III-3 utilized in standard assays in some of the later experiments.

3. Zero-time activated plasma fraction III-3 (ZTPF). Fraction III-3 solutions were activated at the beginning of the sixty minute incubation period of the standard assay by adding 0.5 ml of a fraction III-3 dilution to the fibrin suspension followed immediately with 0.5 ml of the streptokinase solution. The reaction mixture thus had a concentration of 500 Christensen units per ml.

D. Assay of Components Adsorbed on Fibrin

In order to test the tendency of the various fibrinolytic preparations to adsorb on the fibrin suspension, the following procedure was devised. The solution to be tested for adsorbable components and BFS were placed in equal amounts in a fifteen ml centrifuge tube at 0° C. The contents of the tube were frequently mixed by inversion during a thirty minute period after which phosphate buffer was added to the 15 ml mark after centrifugation at 1500 X g at 0° C. for fifteen minutes. The sediment was washed twice and resuspended in 15 ml of phosphate buffer. After the last centrifugation, the fibrin with adsorbed material was resuspended to the original volume in an amount of 0.1 percent Tween 20 phosphate buffer.

for fifteen minutes. The supernatant was transferred to cuvettes for optical density measurements at 280 mu. All measurements were made against a water blank.

B. The BF Unit

An arbitrary unit called the BF (Bovine Fibrin) unit was established to enable comparisons of activities found in the plasma and euglobulin samples. The BF unit is defined as the increase in optical density obtained in the acid extracts of the standard assay with 1 ml of the undiluted plasma or euglobulin as the test solution. Thus if a 1 to 1000 dilution of euglobulin resulted in an optical density .200 greater than that of the control, then the activity of the original euglobulin was $1000 \times .200$ or 200 BF units.

C. Activation of Test Solutions

1. Preactivated plasma and euglobulin. Plasma and euglobulin preparations were mixed with streptokinase solution to give 2,000 Christensen units per ml of mixture. The mixture was incubated in a water bath at 37° C. for fifteen minutes prior to its use in the standard assay as the test solution. In an experiment where more than one concentration of the same plasma or euglobulin were to be tested, the highest concentration required was prepared by the pre-activation method described above and then subsequent dilutions of this activated mixture were made to provide the desired concentrations of plasma or euglobulin for the standard assays.

V. EXPERIMENTS AND DISCUSSION OF RESULTS

A. Preliminary Studies

When streptokinase alone was used as the test solution in standard assays in concentrations ranging from 200 to 1000 Christensen units, no fibrinolytic activity could be detected. This is in agreement with previous reports that streptokinase has no fibrinolytic activity of its own.

The effect of Tween 20 in the BFS preparation was tested by varying its concentration in the suspensions from zero to 1.0 percent and then performing the standard assay with these fibrin suspensions as substrates. ZTPF solution (3 ug per ml reaction mixture) was the test solution for each suspension. There was no significant difference in fibrinolytic activity due to the amount of Tween 20 present. Because reagent blanks increased in optical density from .05 to .15 it was concluded that Tween 20 absorbs light at 280 mu. It was also noted that without Tween 20 in the fibrin suspension, the fibrin had a greater tendency to clump.

In another experiment, stored fibrin suspensions were washed by centrifugation and resuspension in 0.1 percent Tween 20 phosphate buffer from one to eight times. These washed suspensions were then used in standard assays utilizing a constant concentration of ZTPF solution (2.5 ug per ml reaction mixture) as the test solution.

The standard assay was then performed with the fibrin suspension and any adsorbable products accompanying it as the substrate, and with phosphate buffer or streptokinase solution (in the case where activation of the adsorbed material is desirable) substituting for the test solution.

The concentration of the original solution tested for adsorbable components was treated as if it were the concentration of a test solution in a standard assay in calculating the amount of material "made available" for adsorption.

E. Nomenclature

A summary of the original abbreviations utilized in this study is given below:

BFS - Bovine Fibrin Suspension

BF unit - A unit of measurement of specific fibrinolytic activity of plasma and euglobulin samples

PPF - Preactivated Plasma Fraction III-3

LEFS - Lysine-Extracted (Bovine) Fibrin Suspension

ZTPF - Zero-Time (Activated) Plasma Fraction III-3.

No decrease in fibrinolytic response was observed due to the repeated washings.

B. Reproducibility of the Standard Assay

In order to demonstrate the extent of deviation of multiple determinations using the standard assay with bovine fibrin suspension as the substrate, eight assays were performed for each of four ZTPF concentrations (1, 2, 3, and 5 ug per ml reaction mixture). This experiment was also designed to determine the effect of substrate storage on the standard assay. Eight of the assays for each concentration of ZTPF were performed using bovine fibrin suspension stored frozen for six weeks while the remaining eight were performed utilizing freshly prepared bovine fibrin suspension.

The results of these assays are given in Table 1 where the mean value and mean deviation for each concentration of test solution are also given. It is evident that the storage of the fibrin suspension had no detectable effect on its use as a substrate in the assays. Good reproducibility in multiple assays in each of the four concentrations was obtained.

C. Activation of Plasma Fraction III-3

The concentration of streptokinase giving maximum fibrinolytic response from fraction III-3 of a high concentration (8 ug per ml reaction mixture) was detected by varying the amount of streptokinase

Table 1

Reproducibility of the Standard Assay and
Storage Stability of the Substrate

Fraction III-3 Concentration	Bovine Fibrin Suspension	Activity (O.D./60 min.)		Mean Activity	Mean Dev.
	Stored	.087	.095	.099	±.008
		.085	.108		
		.095	.110		
		.095	.110		
1 ug per ml reaction mixture	Fresh	.091	.107	.103	±.009
		.094	.110		
		.094	.112		
		.097	.115		
	Stored	.181	.203	.199	±.010
		.186	.205		
		.191	.211		
		.201	.215		
2 ug per ml reaction mixture	Fresh	.181	.201	.198	±.008
		.185	.205		
		.196	.206		
		.200	.211		
	Stored	.261	.290	.284	±.013
		.261	.297		
		.280	.300		
		.284	.302		
3 ug per ml reaction mixture	Fresh	.262	.290	.285	±.011
		.271	.295		
		.278	.300		
		.280	.304		
	Stored	.359	.380	.378	±.011
		.365	.390		
		.370	.392		
		.375	.395		
5 ug per ml reaction mixture	Fresh	.357	.390	.382	±.013
		.367	.391		
		.374	.395		
		.385	.401		

Standard assays performed with either stored (in frozen state for six weeks) or freshly prepared bovine fibrin suspensions (BFS) as substrates and one of four zero-time activated fraction III-3 (ZTPF) concentrations as the test solution.

FIGURE 2

Streptokinase Activation of Plasma Fraction III-3

× - ZTPF (8 ug/ml reaction mixture)

● - PPF (8 ug/ml reaction mixture)

Activation expressed as Δ O.D./60 min. obtained in the standard assay with indicated plasma fraction preparations as test solutions and BFS as substrate. Streptokinase concentration expressed in Christensen units/ml reaction mixture.

utilized to activate the fraction in standard assays with BFS as substrate. The streptokinase activating the mixtures (either zero-time activation or preactivation) ranged from zero to 500 units per ml of reaction mixture. The results shown in Figure 2 demonstrate that the maximal response was obtained at a streptokinase concentration of about 250 units per ml of reaction mixture for both activation procedures. Since the minimum concentration of streptokinase was 500 units per ml of reaction mixture for all subsequent assays to be presented, that amount of streptokinase was considered sufficient to fully activate the system.

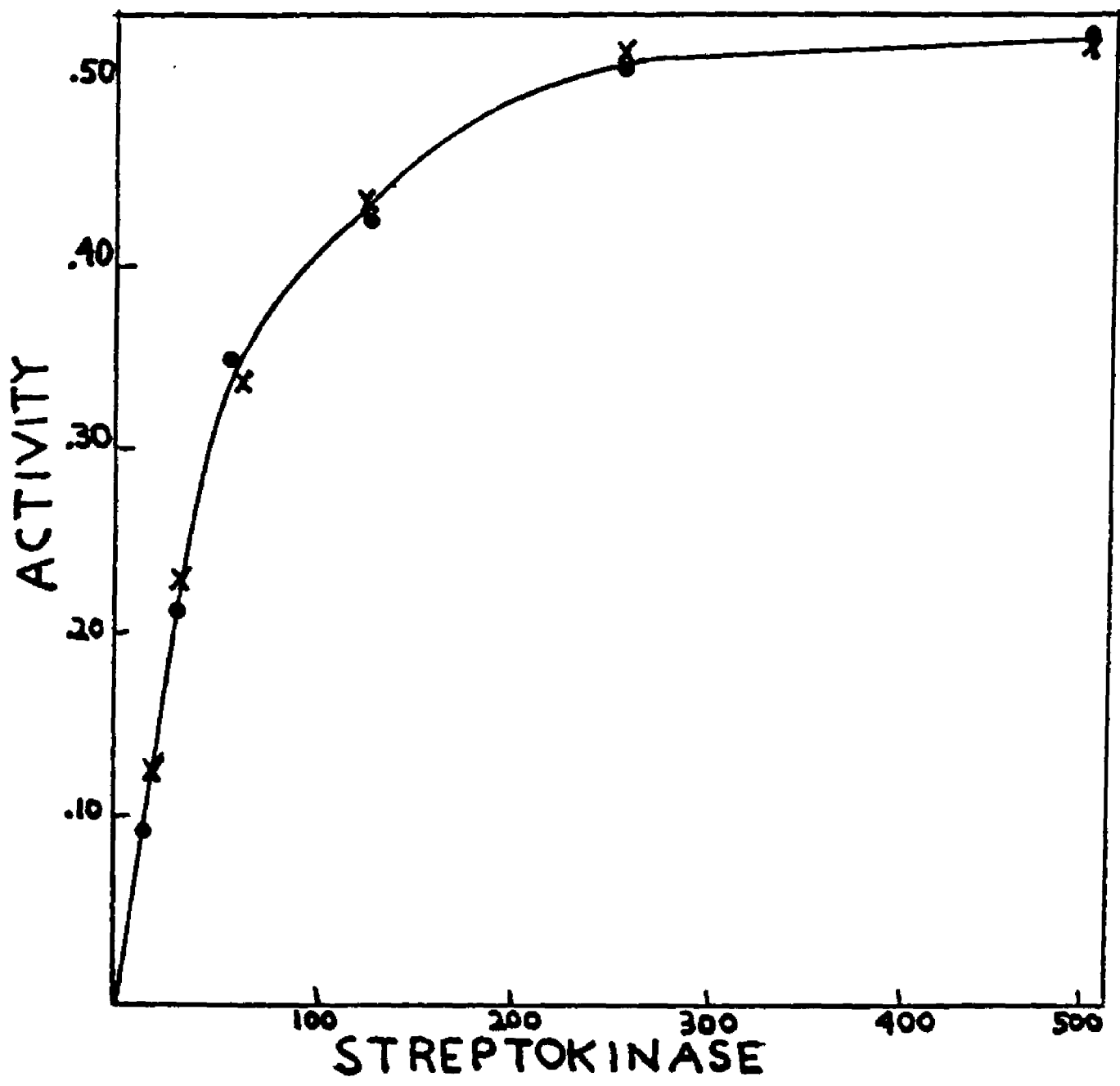
A subsequent experiment was performed with several ZTPF and PPF concentrations (2, 4, and 6 ug per ml reaction mixture) as test solutions and BFS as substrate in standard assays. The zero time activated test solutions and those preactivated with streptokinase (500 units per ml reaction mixture) had the same fibrinolytic activity in the standard assay for each test solution concentration used. It was concluded that, because no increase in response was obtained with preactivated test solutions over those activated at zero time, streptokinase activation of the fraction was very rapid and maximum activity was demonstrated immediately after the addition of the bacterial activator.

D. Component Causing Lysis of Bovine Fibrin

The fibrinolytic activity of streptokinase activated fraction III-3 was due to either the direct action of activated human plasmin

on the fibrin substrate or by streptokinase-induced activation of bovine plasminogen endogenous to the fibrin substrate. The latter sequence would require the presence of a human activator formed by the action of streptokinase on a proactivator present in fraction III-3.

Because endogenous bovine plasminogen is destroyed by heating the fibrin at 80° C., for fifteen minutes (130) a portion of the fibrin suspension was so treated. After cooling the suspension, concentrations (up to 12 ug per ml reaction mixture) were added to aliquots of the treated suspension and assayed using the standard method with BFS as substrate. No fibrinolytic activity could be demonstrated in any of the concentrations tested. This was evidence that streptokinase-induced human plasma activator was the agent causing fibrinolytic activity in the nonheated suspensions. It was noted, however, that after the fibrin suspension was heated its physical appearance changed; the fibrin particles settled rather quickly instead of gradually as before the heat treatment. This possible physical change of the substrate itself could make it a less effective substrate. If this were so, then the lack of response of the suspension after heating may not be due to heat inactivation of endogenous bovine plasminogen, but to denaturation of the fibrin substrate itself. Additional evidence was considered necessary to demonstrate that an activator was indirectly causing lysis of the bovine fibrin suspension.



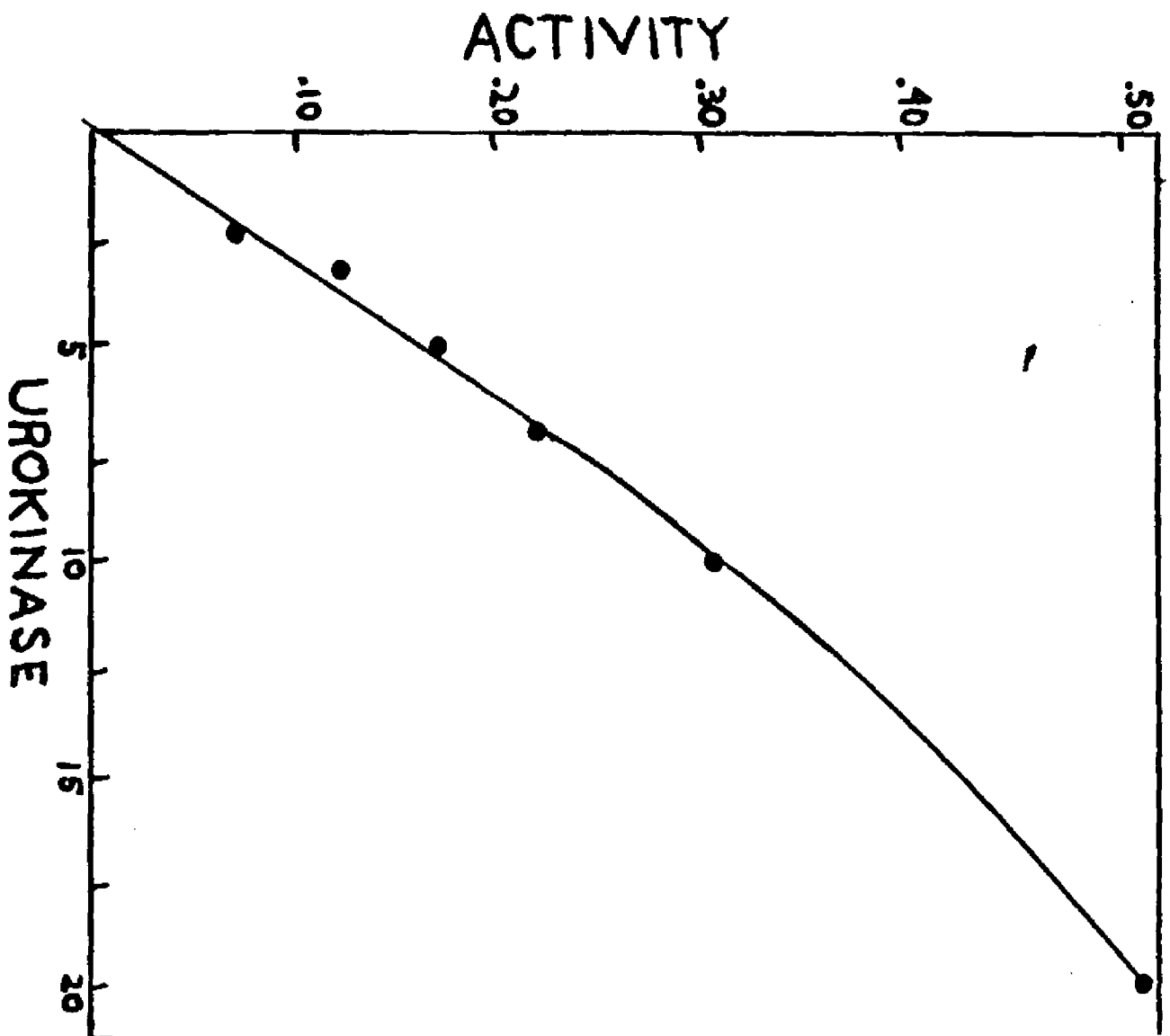
Since urokinase has no fibrinolytic activity, a demonstration of lysis with the addition of urokinase to bovine fibrin suspensions would provide excellent evidence that the urine activator caused fibrinolysis by activating the plasminogen endogenous to the fibrin suspensions. Figure 3 gives the results of an experiment where purified urine activator (urokinase) in varying concentrations was added at zero incubation time to a series of BFS aliquots in the standard assay procedure. The resulting plot shows an initially linear activity response with the slope decreasing in higher concentrations of the activator. The ability of urokinase to induce lysis of the fibrin suspension was considered as direct evidence that the fibrin suspension contained endogenous plasminogen.

The solubilizing effect of lysine solutions on the endogenous plasminogen of human fibrinogen has been previously discussed. If the bovine fibrin suspension extracted with a lysine solution has diminished fibrinolytic response to a streptokinase-activated fraction III-3 solution, then additional evidence would be available demonstrating the presence of endogenous plasminogen in the fully responsive nonextracted fibrin suspension. In order to test the effect of lysine solutions on the fibrin suspension, the following experiment was performed. Lysine solutions ranging from $.01 \text{ M}$ to 0.1 M were used to wash aliquots of the BFS preparation in the manner previously described in the preparation of lysine-extracted bovine fibrin suspension (LEFS). A BFS aliquot was treated with distilled water instead of a lysine

FIGURE 3

Urokinase-Induced Fibrinolytic Activity

Activity expressed in Δ O.D./60 min. obtained in the standard assay with urokinase dilutions as test solutions and BFS as substrate. Urokinase concentration expressed as percent concentration (v/v) purified urine activator in the reaction mixture.



solution as a control. Standard assays were performed with the extracted fibrin suspensions and a constant amount of ZTPF solution (10 ug per ml reaction mixture) for each assay. The results of the experiment are shown in Figure 4. It is evident that decreased fibrinolysis occurred with increasing lysine concentration in the extracting solutions. The experimental results indicate that as the amount of endogenous plasminogen was progressively removed with increasing lysine concentration in the extracting solutions, the lytic activity induced by the addition of streptokinase activated fraction III-3 decreased and eventually approached zero activity.

From the above experiments it was concluded that the fibrinolytic activity of activated fraction III-3, in the concentrations tested, was not due to its plasmin, but to its activator which was formed in the presence of streptokinase and which activated endogenous plasminogen in the fibrin suspension. Thus, the BFS preparation provided a substrate whose fibrinolytic response depended on the concentration of activator in the plasma fraction.

It is interesting to note that repeated washings of the fibrin suspension with phosphate buffer did not reduce endogenous plasminogen levels to a point where fibrinolytic response with streptokinase induced plasma activator was affected. This observation suggests that the plasminogen in the suspension has a great affinity for fibrin and is not easily removed.

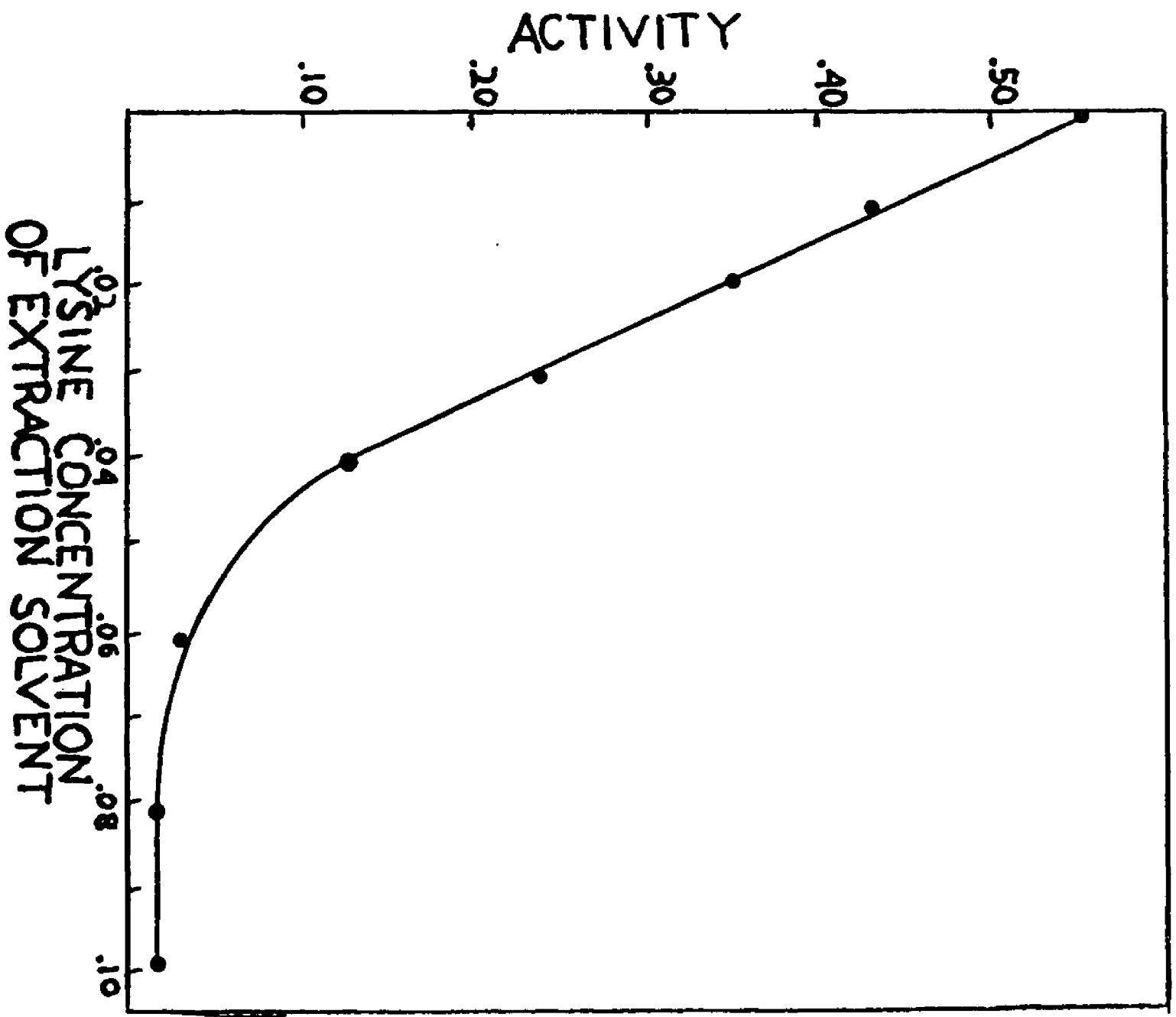


FIGURE 4

Fibrinolytic Response of Bovine Fibrin
Extracted with Lysine Solutions

Activity expressed as Δ O.D./60 min. obtained in the standard assay with fibrin suspensions extracted with indicated lysine solutions as substrates and a constant amount of activator (10 ug ZTPF/ml reaction mixture) as test solution. Lysine concentration expressed in molarity.

FIGURE 5

Rate of Plasma Activator-Induced Fibrinolysis

- - 1.5 ug ZTPF/ml reaction mixture
- X - 3.0 ug ZTPF/ml reaction mixture

Δ O.D. at 280 mu of trichloroacetic acid extracts obtained after various incubation times of a reaction mixture was measured. The reaction mixture contained indicated ZTPF concentration and 15 mg BFS/ml.

E. Kinetic Studies of Plasma Activator

The component in streptokinase-activated fraction III-3 producing fibrinolytic activity in BFS preparations was considered an activator whose effect was on plasminogen present in the fibrin. In order to study the fibrinolytic rate of the reaction occurring on addition of plasma activator (in streptokinase-activated fraction III-3) to BFS the following experiment was performed. To a volume of BFS in a 50 ml Erlenmeyer flask (in the Dubnoff incubator-shaker) was added half a volume of a fraction III-3 solution followed quickly by half a volume of a streptokinase solution (4,000 units per ml). Immediately after streptokinase addition and shaking initiated, a 2.0 ml aliquot of the reaction mixture was removed and placed in a centrifuge tube containing 2.0 ml of 10 percent trichloroacetic acid. This was the zero incubation time assay. At given time intervals up to 120 minutes, 2.0 ml aliquots were similarly removed and treated. The processing of the trichloroacetic acid extracts and the measurement of acid-soluble fibrinolytic products followed the procedure described for the standard assay. The entire procedure described above was performed on two fraction III-3 concentrations (1.5 and 3.0 ug per ml reaction mixture). The results of the experiment are shown in Figure 5. It is evident from these results that both concentrations of activator tested demonstrated a linear increase of spectrophotometrically-measurable acid-soluble fibrinolytic products. Apparently activator formed in the first minute or two of the reaction immediately activated a specific amount of

plasminogen in the suspension. This observation suggests that streptokinase-induced activator forms plasmin stoichiometrically and not enzymatically during the test period. This observation is in agreement with a report in which heat of activation studies indicated streptokinase-induced plasma activation of a non-enzymatic nature while urokinase activation was enzymatic (70). Earlier studies indicated, however, that the activator formed plasmin enzymatically from plasminogen (casein and synthetic esters substrates) (11). Figure 5 illustrates the first order kinetics that were observed in regard to activator concentration. The rate of the reaction with the higher concentration of fraction III-3 (3.0 ug per ml of reaction mixture) was slightly less than twice the rate at the lower concentration (3.0 ug per ml).

The experimental observations discussed above indicate that the stoichiometric formation of plasmin by the activator results in a fibrinolytic activity dependent upon the amount of activator initially present in the test solution. In order to obtain more information on this process, the reciprocal plot method of Lineweaver and Burk (134) was applied to results obtained in the following experiment. Four concentrations of activator (ZTPF) were tested for their fibrinolytic inducing activity on suboptimal concentrations of BFS. The four activator concentrations tested were 1.5, 2.0, 2.5 and 3.0 ug plasma fraction III-3 per ml of reaction mixture. Except for the lower fibrin concentrations, the standard assay was used to obtain the

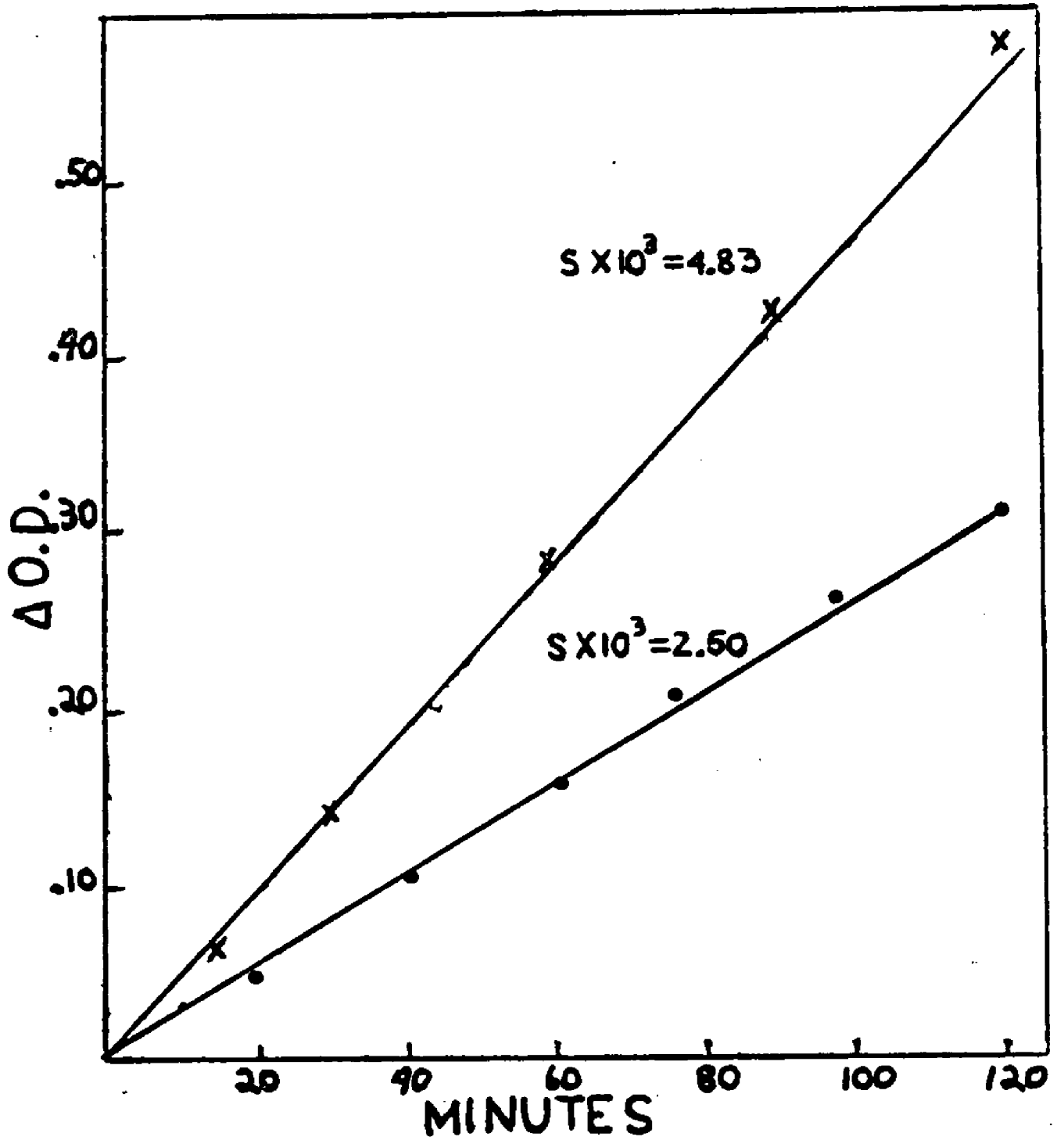


FIGURE 6

Effect of Substrate Concentration on Plasma
Activator-Induced Fibrinolytic Activity

S - BFS in mgs/ml reaction mixture

V - Δ O.D./60 min. obtained in procedure following that of the standard assay with indicated BFS concentrations as substrates and PPF dilutions as test solutions.

	Activator Conc.*	Calculated K_m (mgs/ml)	Calculated V_{max}
A	1.5	2.6	.15
B	2.0	2.6	.22
C	2.5	2.7	.26
D	3.0	3.0	.30

*Activator concentration expressed as ug/ml reaction mixture of PPF

velocity as the increase in optical density of trichloroacetic extracts of the reaction mixtures after an incubation time of 60 minutes. The results of the experiment are shown in Figure 6. A linear relationship was obtained for each concentration of activator. These results imply that, with a constant amount of activator, the amount of plasmin formed was also constant for all substrate concentrations tested and the velocity is then dependent on the substrate concentration alone. The results, therefore, provide evidence that the amount of endogenous plasminogen is not rate limiting in the substrate concentrations tested. The apparent K_m values, calculated from the abscissa intercepts, agreed except for the value obtained with the highest activator concentration (3.0 ug per ml). The theoretical maximum velocities (V_{max}) calculated from the ordinate intercepts are also given in Figure 6.

An experiment was designed to relate fibrinolytic activity to concentration of the activator. The standard assay was performed in which different concentrations of ZTPF served as the activator sources and as test solutions with BFS as substrate. Several nonactivated concentrations of fraction III-3 were assayed in the same manner. The results of this experiment are shown in Figure 7. It is evident from these results that, initially, the lytic activity increased linearly with activator concentration but became nonlinear and less responsive in activator concentrations above 3 ug per ml reaction mixture. As discussed in Chapter II, plasmin may act as its own substrate (auto-catalysis) and the suppressed increase in activity in

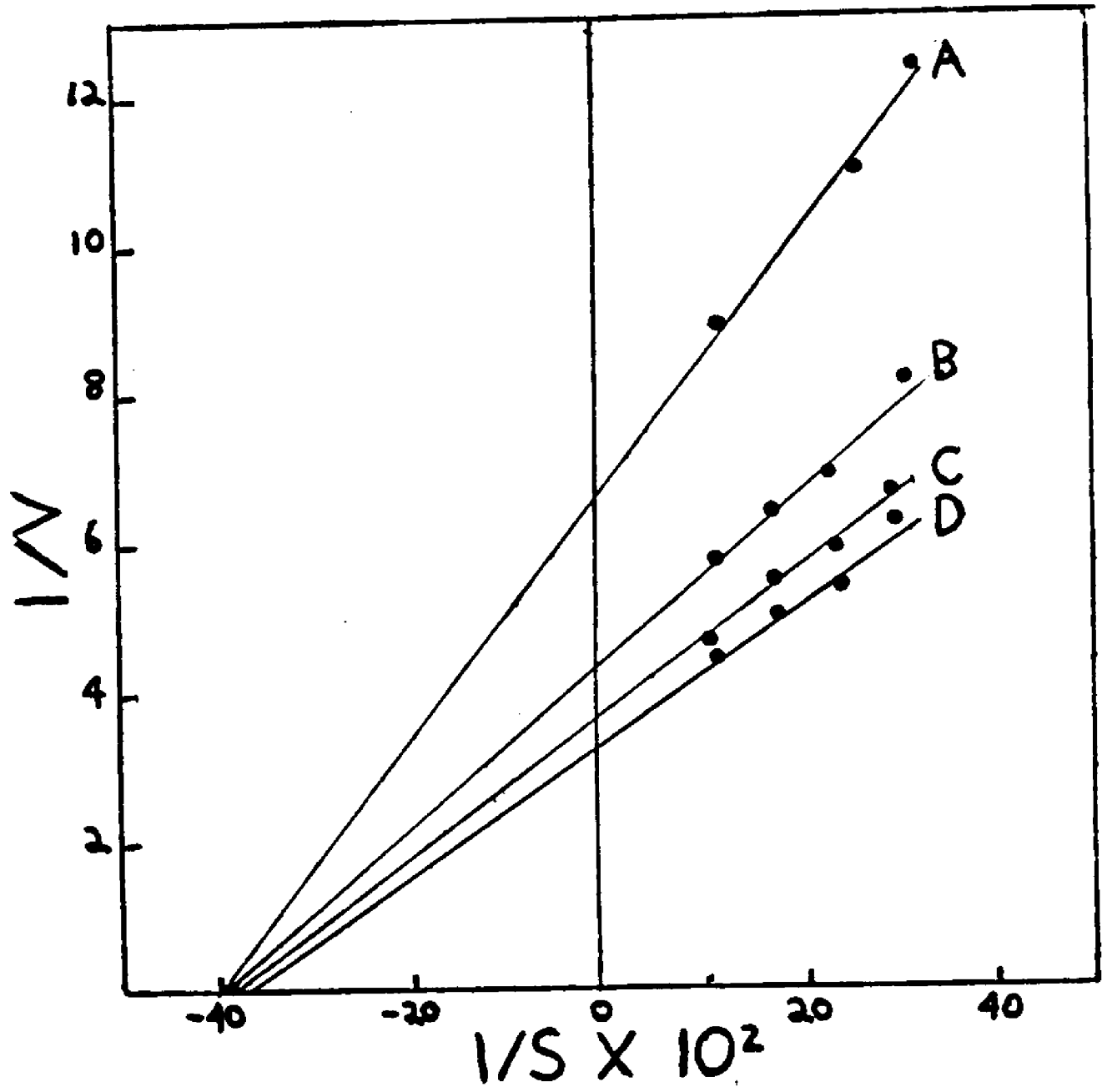


FIGURE 7

Fibrinolytic Activity Induced by Plasma Activator

- - theoretical maximum velocities obtained in Figure 6
- - experimental values with ZTPF as test solution
- × - experimental values with nonactivated fraction III-3 as test solution

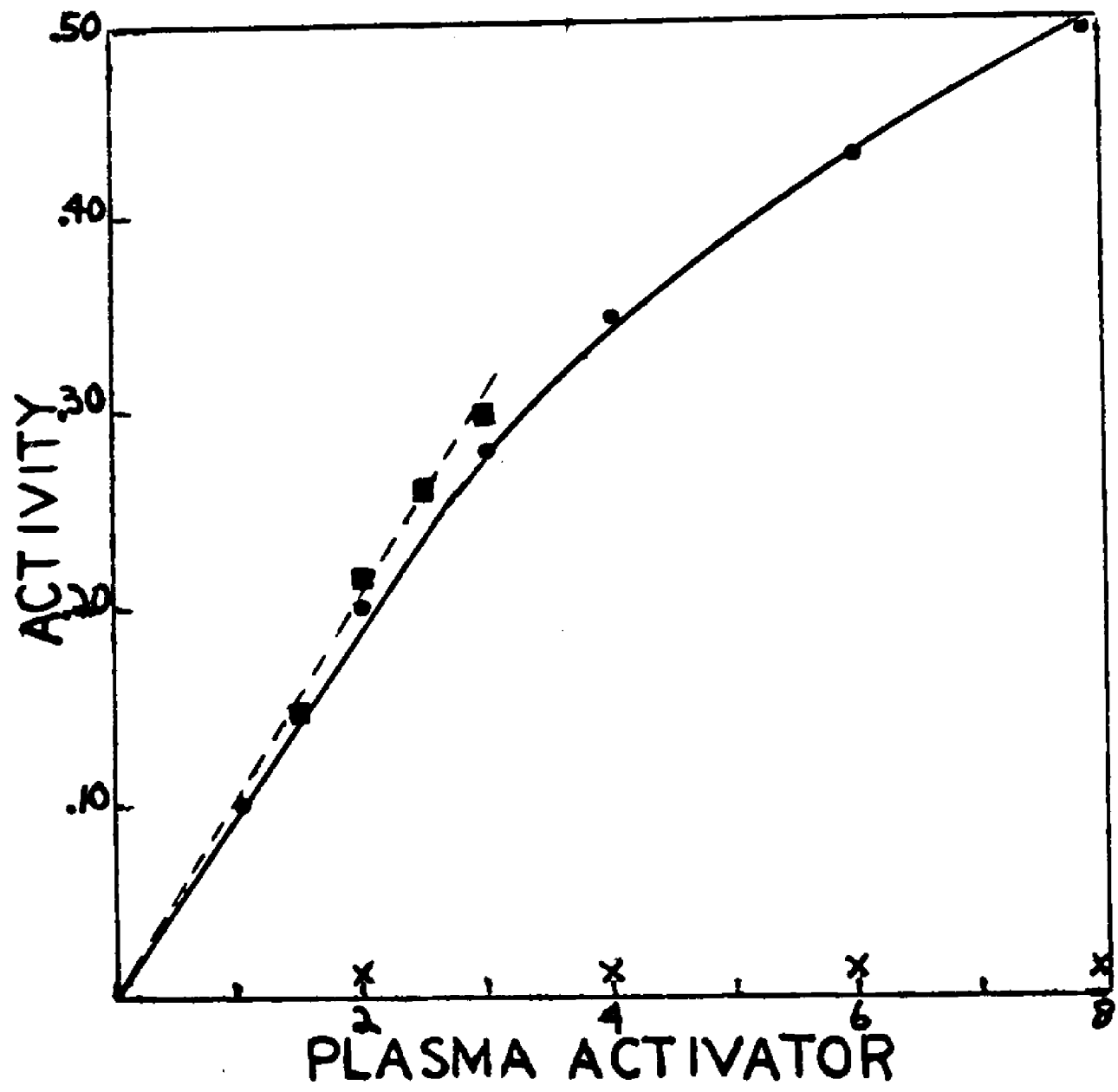
Activity expressed as Δ O.D./60 min. obtained in a standard assay with indicated activator preparations as test solutions with BFS as substrate. Plasma activator concentration expressed as ug/ml reaction mixture of fraction III-3.

the higher (above 3 ug per ml) activator concentrations may be due to a plasmin-plasmin interaction of the activated endogenous bovine plasminogen.

F. Adsorption of Activators and Proactivator

Spontaneous blood activator (such as the type detected after severe exercise) and the streptokinase-induced activator have been reported to be totally adsorbed to fibrin during coagulation of a fibrinogen-activator mixture (90). About 35 percent of the pro-activator and practically no urokinase are adsorbed. Exposure of activator to preformed clots, however, resulted in very little adsorption. These observations suggested that activator binding was due to the "entrapment" of activator in the fibrin matrix during the polymerization step and not due to surface adsorptive properties of the fibrin polymer after it was formed.

In order to study the affinity of activators and proactivator for the fibrin suspension, the following experiment was performed. Several concentrations of urine activator as well as of nonactivated and preactivated fraction III-3 were mixed with aliquots of BFS after which the suspensions were thoroughly washed and used in standard assays. The entire adsorption procedure as described in Chapter V was used. For the urokinase and activated fraction III-3 adsorption assays, 1.0 ml aliquots of phosphate buffer were used as test solutions. In the assays for testing the adsorption of proactivator from



nonactivated fraction III-3 solutions, 1.0 ml aliquots of streptokinase solution (2,000 units per ml) were used. This latter procedure permitted the transformation of all adsorbed proactivator to activator. The results of the experiment are shown in Figure 8. By comparing the adsorbed activity of BFS exposed to proactivator and activator solutions (Figure 8) with the activity of the same concentrations of proactivator and activator in the test solutions in standard assays (Figure 7), it is evident that total adsorption took place. The activity adsorbed to fibrin was equal to the maximum activity that could be obtained. Adsorbed urokinase activity was less than 10 percent of the activity expected if total adsorption took place (Figure 3). Proactivator and activator thus have a greater affinity for the fibrin surface than urokinase. Activator and proactivator may possibly be adsorbed on the fibrin surface wherever an endogenous plasminogen molecule was present and thus form a stoichiometric complex with the proenzyme. It is interesting that proactivator has an affinity for the fibrin as does activator. The study with urokinase adsorption indicated a different mechanism since no great affinity for the fibrin surface was observed. The adsorption studies indicated that streptokinase-induced activator and proactivator were readily adsorbed on preformed fibrin when the fibrin surface area has been increased. This experimental observation does not agree with the previously discussed concept of activator "entrapment" in the forming clot.

FIGURE 8

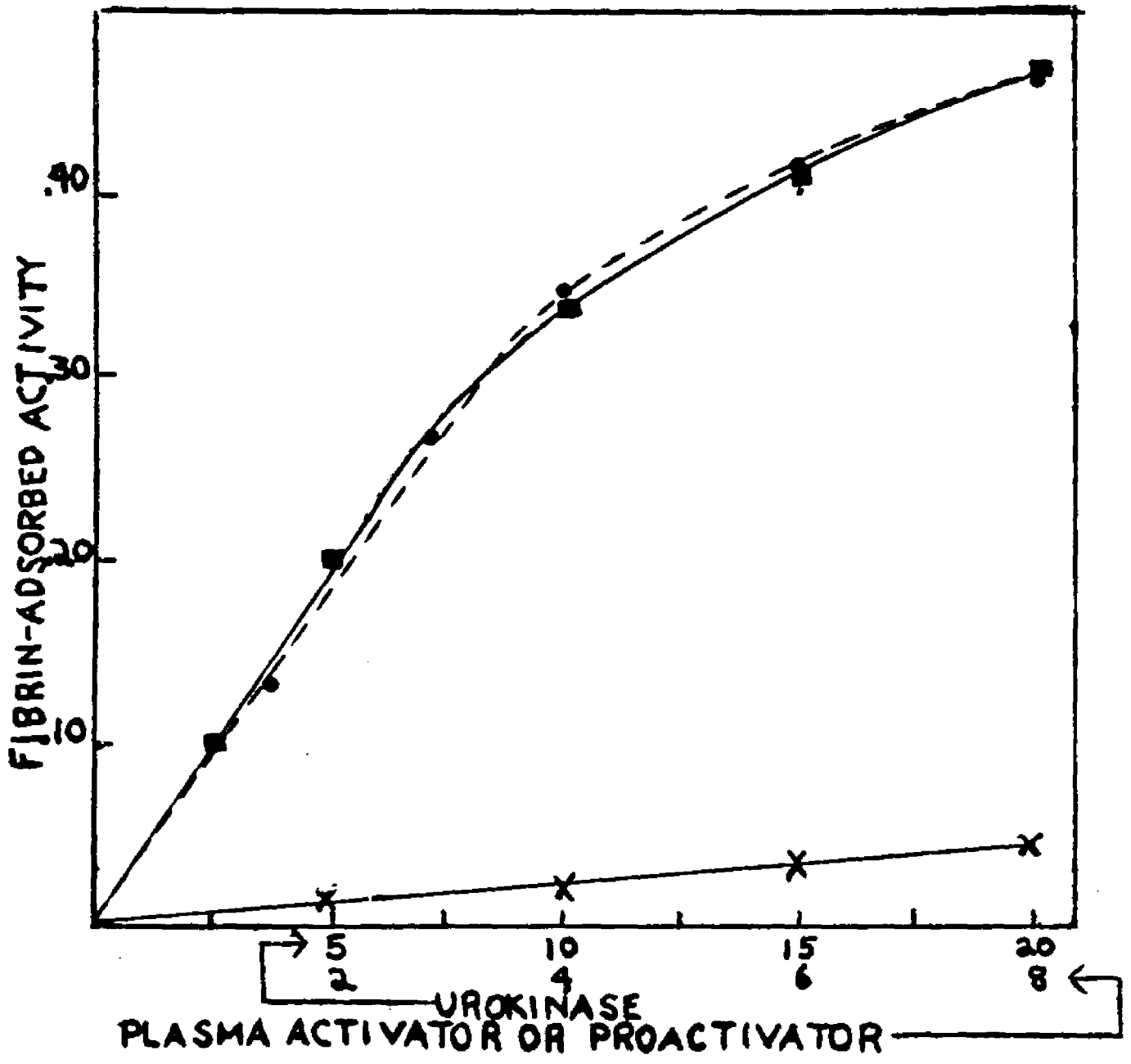
Activity Induced by Adsorbed Activator or Proactivator

X - Urokinase, ■ - Plasma Activator, ● - Plasma Proactivator

Fibrin-adsorbed activity expressed as Δ O.D./60 min. obtained in the standard assay with BFS (with any adsorbed components) as substrate. Activator concentrations were those provided for adsorption. Urokinase concentration expressed in percent concentration (v/v) purified urine activator in reaction mixture. Plasma activator (PPF) and proactivator (fraction III-3 activated after adsorption procedure) concentrations expressed in ug/ml reaction mixture.

G. Fibrinolytic Rate of Urokinase

Since plasma activator and proactivator have an affinity for fibrin, while the urine activator, urokinase, has very little, a different activation process for the endogenous plasminogen seemed possible. In order to compare the fibrinolytic rates previously obtained for plasma activator with those obtained with urokinase, the experimental method previously used for the plasma activator analysis was used to study the fibrinolytic rate for two purified urine activator concentrations (2.5 and 5.0 volume percent in reaction mixtures). The results of the urine activator rate study appear in Figure 9. The rates for the two urokinase concentrations tested were distinctly nonlinear. This is in contrast to the rates obtained with the plasma activator. Plasminogen appears to be continually activated during the incubation period and thus the rate increases during the course of the experiment. Urokinase activation results, therefore, are those expected of an enzyme (urokinase) activating a proenzyme (plasminogen) with the assay depending on the enzymatic behavior of the final activation product (plasmin). An alternate explanation is that an initial inductive period was required before the full activation effect of urokinase was elicited. This mechanism would evoke a biphasic rate curve similar to the curves in Figure 9. By noting the differences in the rates (Figure 5 and 9) and in the adsorption characteristics (Figure 8), an apparent difference in the mechanism of endogenous plasminogen activation was observed.



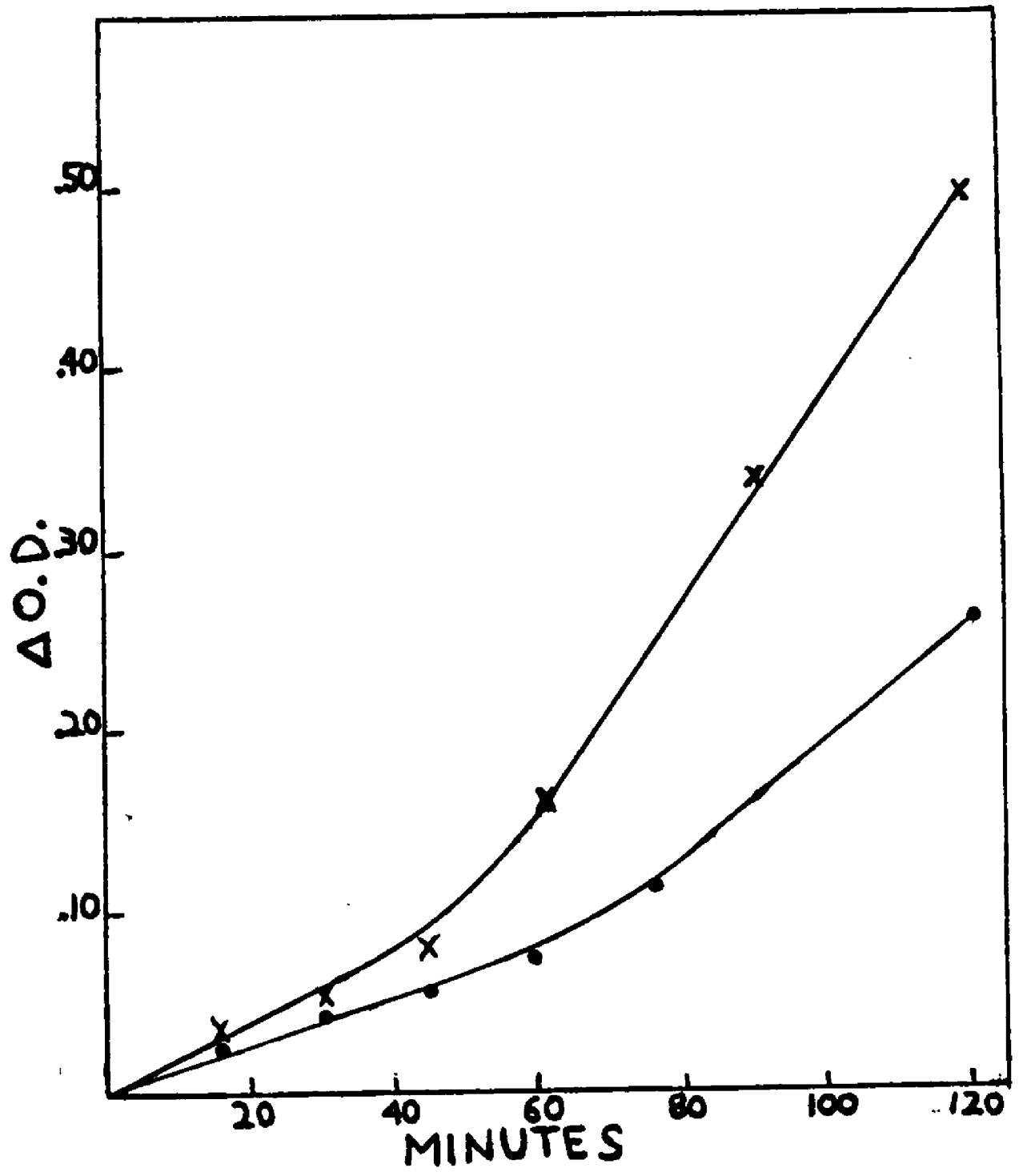


FIGURE 9

Rate of Urokinase-Induced Fibrinolysis

● - 2.5%, X - 5.0% urokinase concentrations expressed as the percent concentration (v/v) of purified urine activator in the reaction mixture. Δ O.D. at 280 m μ of trichloroacetic acid extracts obtained after various incubation times of a reaction mixture was measured. The reaction mixture contained indicated urine activator concentration and 15 mg BFS/ml.

H. Activator in Plasma and Euglobulin

In all the previous experiments the activator source was either streptokinase-activated fraction III-3 or urokinase. It was considered essential that plasma and the euglobulin derived therefrom be tested in order to measure the extent of their ability to activate the endogenous plasminogen in BFS. In the first experiment, a plasma and its euglobulin fraction (Sample 1) prepared by the method of Kowalski (45) were tested in various dilutions for fibrinolytic activity by the standard assay after preactivation with streptokinase. Several assays were performed on dilutions which were not activated in order to detect spontaneous activator in the test solutions. The results of the experiment appear in Figures 10 and 11. The euglobulin activities were initially linear with maximum response to euglobulin concentration followed by a comparatively lower activity response in higher euglobulin concentrations. This phenomenon was quite similar to that obtained with activated fraction III-3 (Figure 7). The plasma activity was initially greater than that of its euglobulin fraction in the lower concentrations tested. In the region of linear response (in low concentrations) plasma activator fibrinolytic activity was higher than that of its euglobulin fraction. In the more concentrated plasma and euglobulin solutions tested, plasma activity approached a maximum while that of the euglobulin continued to rise though at a decreased rate.

FIGURE 10

Effect of Plasma Activator Concentration
on Fibrinolytic Activity

- - preactivated plasma, X - preactivated euglobulin
■ - nonactivated plasma, ▲ - nonactivated euglobulin

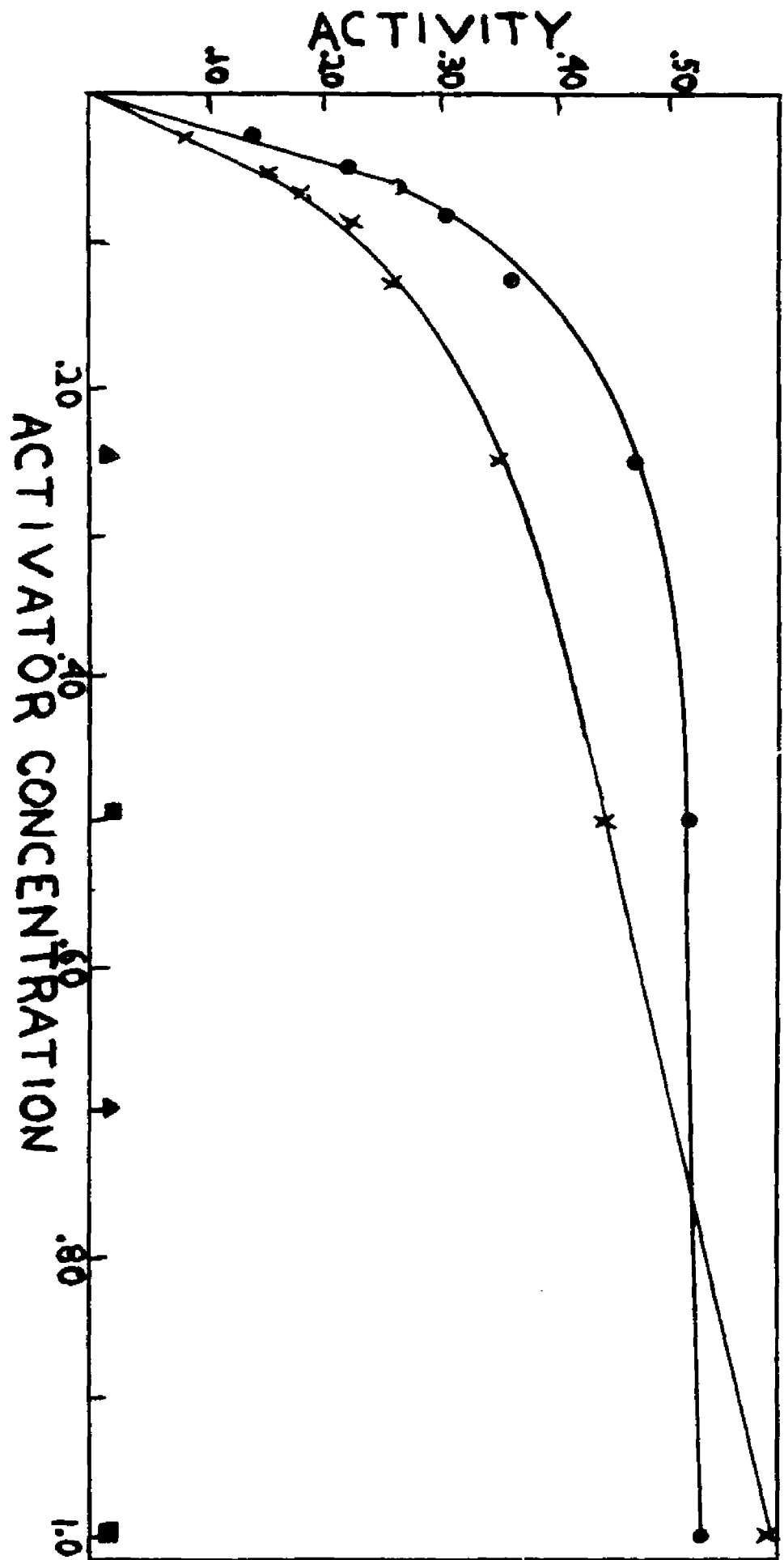
Activity expressed as Δ O.D./60 min. obtained in a standard assay with indicated plasma or euglobulin dilutions as test solutions and BFS as substrate. Concentrations expressed as percent concentration (v/v) in reaction mixture.

FIGURE 11

Specific Plasma and Euglobulin Activator Activity

● - preactivated plasma, X - preactivated euglobulin

Concentrations of plasma and euglobulin expressed as percent concentration (v/v) in reaction mixture. BF units calculated from values plotted in Figure 10. The specific activities obtained in the low concentrations are also plotted in insert with expanded concentration scale while the BF units scale is the same.



Because the plasma activator activities were greater than those of its euglobulin in the concentrations where maximum and linear response was obtained, it is assumed that the euglobulin fraction did not contain all of the activator of whole plasma. The more abrupt suppression in fibrinolytic response with increasing concentrations of plasma as compared to euglobulin may be due to the presence of inhibitors in the former. These inhibitors in plasma apparently reduce the activity at a plasma concentration of .20 volume percent or higher while the euglobulin activator activity continues to increase. At concentrations below .10 volume percent the plasma inhibitor effect appears to be diluted out. It was considered significant that the nonactivated plasma and euglobulin concentrations tested had no activity. Spontaneous activator was apparently not present in measurable amounts.

In order to show the effect of dilution on specific activity, the fibrinolytic velocities obtained for the various plasma and euglobulin concentrations shown in Figure 10 were used to calculate BF units of activity. These values were plotted against concentration in Figure 11. Where fibrinolytic response was linear with concentrations, the specific activity was at a maximum value. As the concentration was increased specific activity decreased and approached a minimum value.

Seven other plasma samples and their euglobulin fractions were tested in the same manner as described for the plasma and euglobulin.

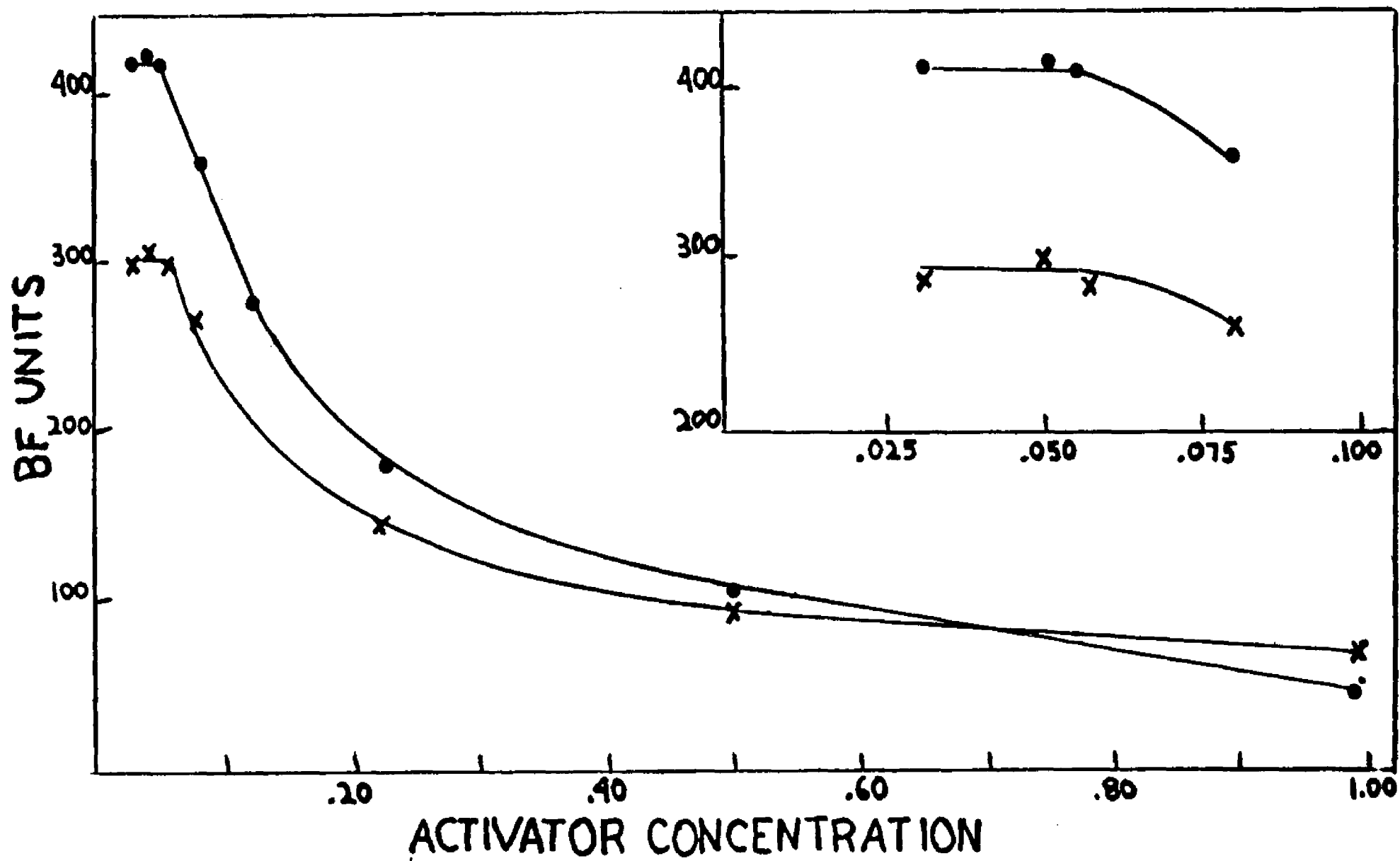


Table 2

Plasma and Euglobulin Total Activator Activity

Sample	1	2	3	4	5	6	7	8
Euglobulin	296	370	248	312	190	270	200	360
Plasma	418	470	560	572	490	488	405	500
Euglobulin/Plasma Activity Ratio	.71	.79	.44	.55	.39	.55	.49	.72

Euglobulins prepared by Kowalski procedure (45). Activities expressed in BF units calculated from values obtained with the standard assay of euglobulin and plasma samples diluted to the range where fibrinolytic response was proportional to concentration. Bovine fibrin suspension (BFS) was the substrate.

The same curve characteristics as those seen in Figures 10 and 11 were obtained when the activities were plotted though quantitative differences were apparent. The plasma and euglobulin values for all eight samples are given in Table 2. It was noted that none of the samples had any significant spontaneous activator activity. The specific activity listed in the table are those obtained with preactivated plasma and euglobulin samples. In every case the total activator activity of plasma was greater than that of its euglobulin fraction. Though no previous study was available for a comparison of values of plasma and euglobulin total activator activity, it has been reported that the chylomicron fraction of plasma contains activator activity after treatment with streptokinase (135). The use of the euglobulin fraction as the test solution for total plasma proactivator and activator properties may thus be subject to serious objections.

I. The Assay of Plasmin

Lysine-extracted fibrin suspension was inactive to streptokinase-induced plasma activator because endogenous bovine plasminogen was not present in the fibrin and, therefore, plasmin activity could not be generated by the activator. In a preliminary experiment it was noted that lysine-extracted fibrin suspension was completely unreactive to purified urine activator in concentrations up to 50 percent in reaction mixtures of standard assays. This observation provided further evidence that lysine-extracted fibrin suspensions were not lysed by

FIGURE 12

Plasmin Fibrinolytic Rate

- - 150 ug PPF/ml reaction mixture
- X - 300 ug PPF/ml reaction mixture

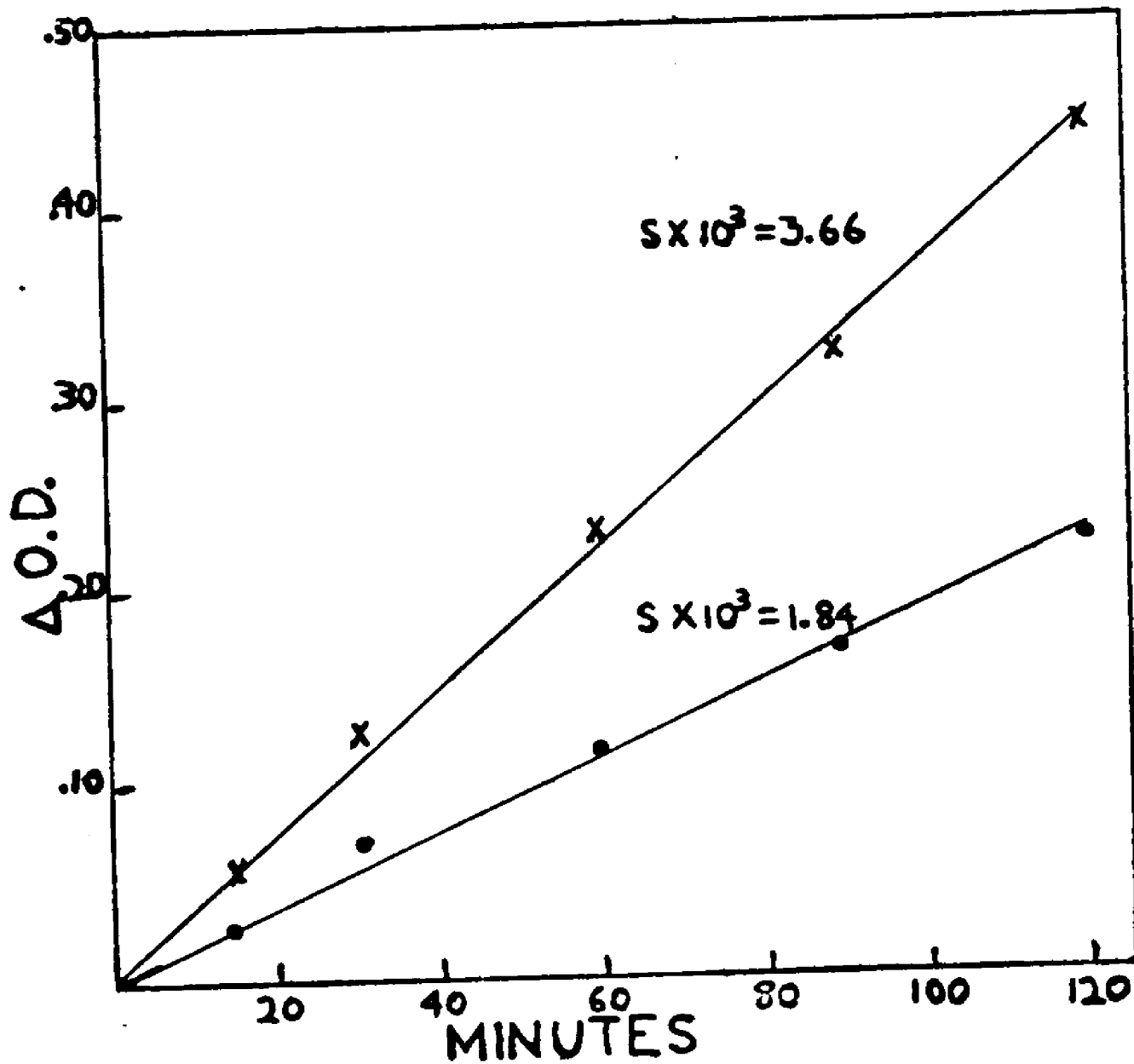
△ O.D. at 280 mu of trichloroacetic acid extracts obtained after various incubation periods of a reaction mixture was measured. The reaction mixture contained indicated PPF concentration and 15 mg BFS/ml.

the indirect action of activators. Since lysine-extracted bovine fibrin suspension was demonstrated to be free of endogenous plasminogen, it was considered to be a suitable substrate for human plasmin.

An experiment was designed to study the rate of fibrinolysis of lysine-extracted fibrin suspensions with two concentrations of PPF (150 and 300 ug per ml reaction mixture) as test solutions. The same method was used as described in the fibrinolytic rate studies of plasma activator, except that lysine-extracted fibrin suspension (LEFS) was the substrate. The results are given in Figure 12. First order kinetics were observed in the two concentrations tested. Approximately a 150 fold increase in activated fraction III-3 was necessary to obtain a response equivalent to that obtained in the activator study with the unextracted fibrin suspension as the substrate. An explanation for the higher concentration requirements necessary to obtain measurable results in assays of plasmin activity may be that the potential activator (proactivator) concentration may be much higher than the potential plasmin concentration in the plasma fraction. The activator assay depends on plasmin present within the fibrin particles while the plasmin assay depends completely on the enzyme in the fluid phase. It is thus a distinct possibility that the former is a much more efficient fibrinolytic system causing a more sensitive measurement of activator than of exogenous plasmin.

Another experiment was performed in which the fibrinolytic activities of plasmin in several concentrations of PPF were obtained with standard assays using LEFS as substrate. Also assayed in the same manner were several concentrations of nonactivated fraction III-3. These results are given in Figure 13. The activity remained proportional to the concentration of enzyme up to a concentration of 300 ug per ml of reaction mixture. Nonactivated fraction III-3 contained a significant amount of plasmin activity. The latter observation was interesting since it indicated that plasmin was present in a nonactivated preparation which had no measurable spontaneous activator. The mechanism by which the high percentage of plasmin activity was formed in nonactivated fraction III-3 was not investigated in this study, but it is well known that plasminogen can readily undergo slow spontaneous activation.

Since plasma is known to contain several plasmin inhibitors, it was considered a poor material to use for total plasmin assays. Plasma euglobulin fractions, however, are acceptable. In the next experiment, therefore, the plasmin activity of a euglobulin preparation (from plasma sample 9) was measured after preactivation, at several different concentrations. The standard assay, with LEFS as substrate, was used to measure the activities. The results of these assays appear in Figure 14. A linear response similar to that obtained with the plasmin assays performed on activated fraction III-3 was obtained. It was concluded that, in the range tested, the



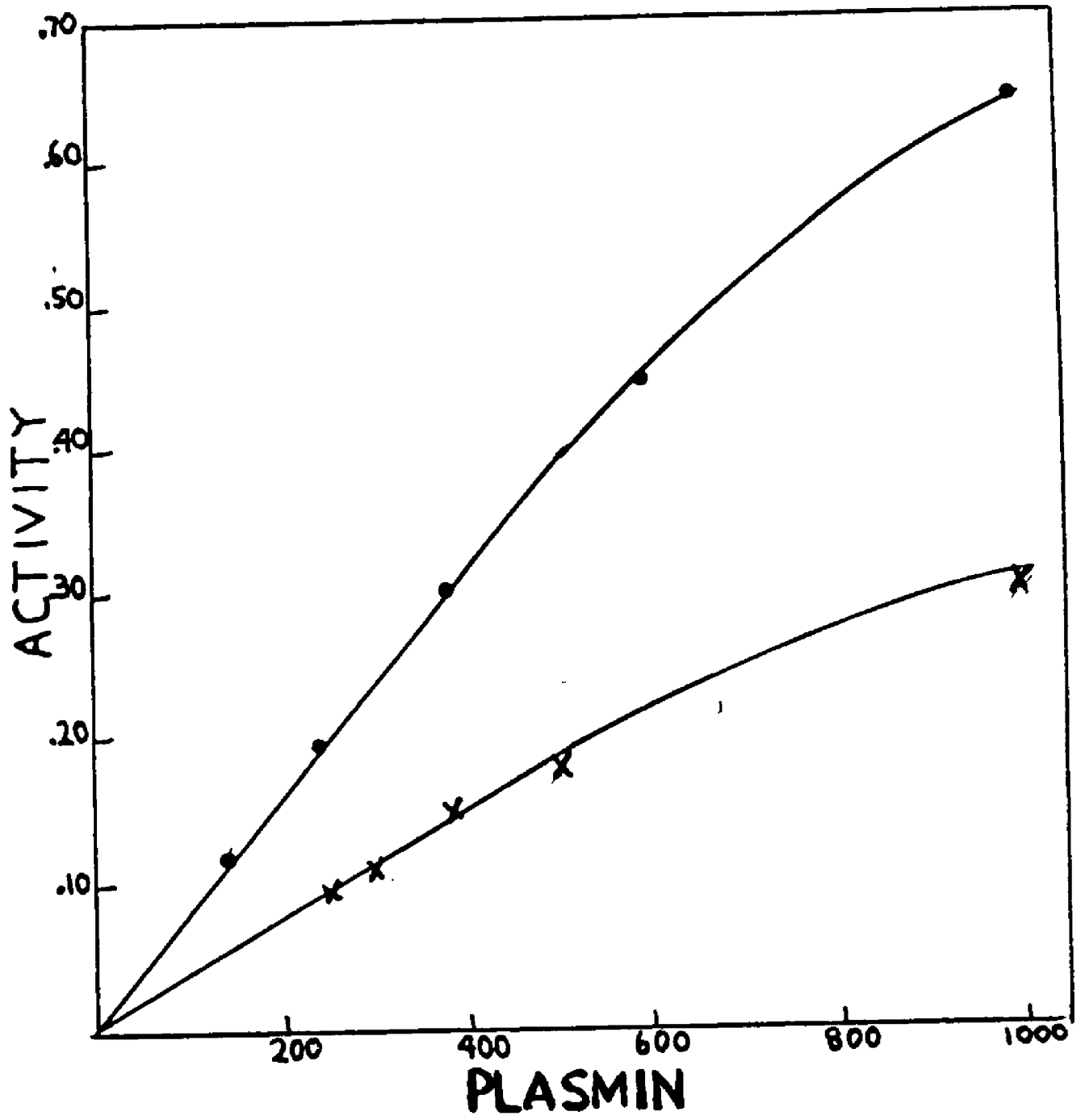


FIGURE 13

Fibrinolytic Activity of Plasmin

- - PPF as test solution
- X - nonactivated fraction III-3 as test solution

Activity expressed as Δ O.D./60 min. obtained in a standard assay with indicated fraction III-3 preparation as test solution and LEFS as substrate. Plasmin concentration expressed in ug fraction III-3/ml reaction mixture.

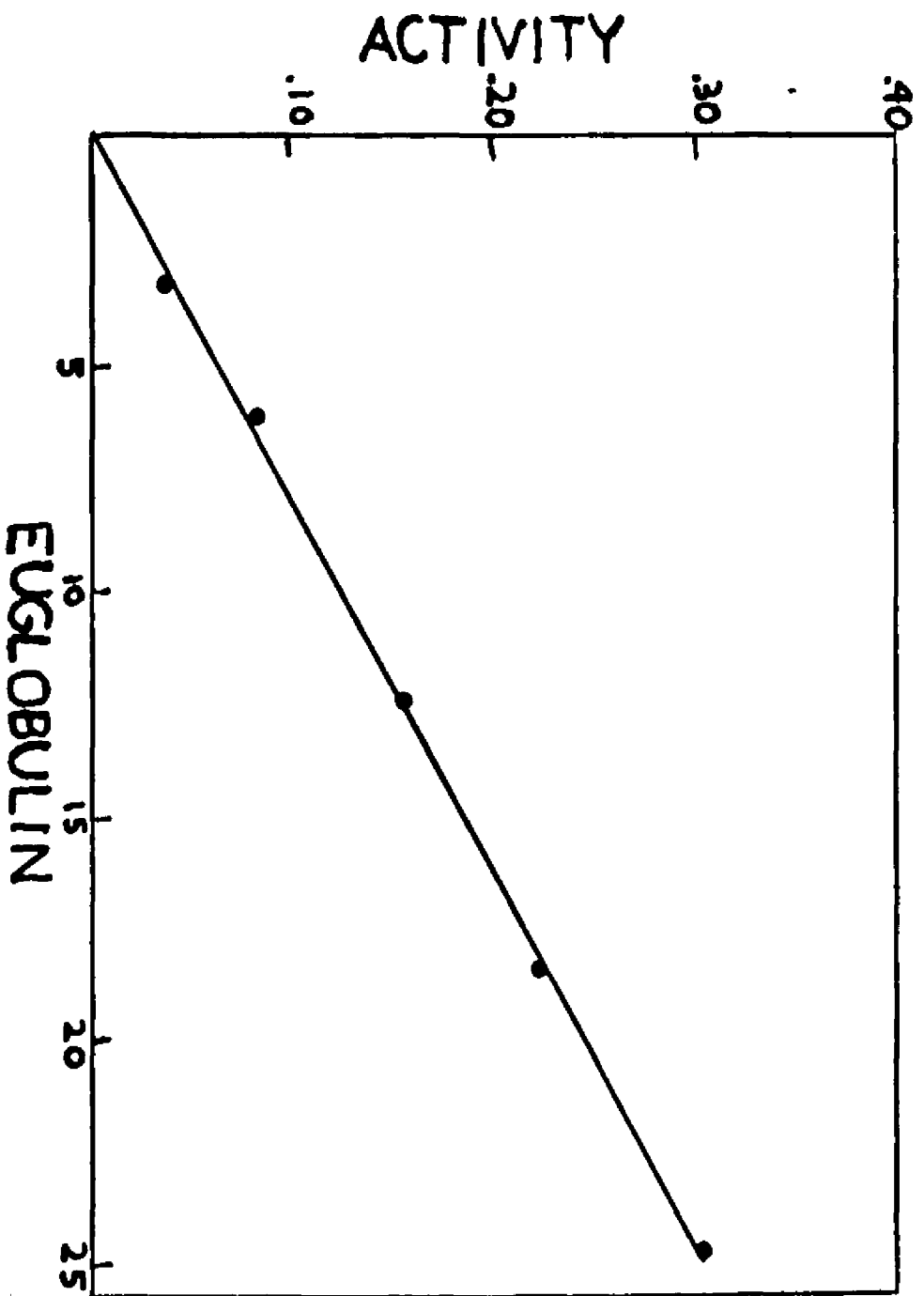


FIGURE 14

Euglobulin Plasmin Activity

Activity expressed as Δ O.D./60 min. obtained in a standard assay with preactivated euglobulin dilutions as test solutions and LEFS as substrate. Euglobulin concentration expressed as percent concentration (v/v) euglobulin in the reaction mixture.

solutions is the activity of plasminogen. The assay system is presented in Table 3. It is seen that a series of four standard assays A, B, C and D will permit complete assay of the individual fibrinolytic components of the test solution. With assays A and B plasmin and plasminogen are measured. The lytic activity with assay A is due to preformed plasmin (spontaneous plasmin) while that of assay B is due to preformed plasmin plus plasminogen activated in the presence of streptokinase (total plasmin). With assays C and D activator and proactivator are measured. The lytic activity detected with assay C is due to preformed activator (spontaneous activator) plus preformed plasmin if one or both of these entities are present in the test solution. The lytic activity obtained with assay D is primarily due to activator formed in the presence of streptokinase plus preformed activator (total activator). A minor percentage of the activity is due to the total plasmin in the test solution. The lytic effect of this exogenous plasmin in assay D is insignificant because of the low plasmin activity present in the highly diluted euglobulin used in this assay.

A series of eight euglobulins (prepared from plasma samples 9 through 16) were tested with the assay system described above. The euglobulin preparations were diluted to give the following concentrations of euglobulin in the reaction mixtures (expressed as volume percent): assays A and C, 25 percent; assay B, 12.5 percent, and assay D, 0.1 percent. In assays B and D preactivated test solutions

euglobulin fraction plasmin fibrinolytic activity was proportional to the concentration of the fraction in the reaction mixtures and could be used to calculate specific plasmin activity as BF units.

J. Total Assay of Fibrinolytic Components

Based on the experimental evidence presented, the conclusion is reached that BFS can be used to assay activators and LEFS to assay plasmin. BFS is responsive to activators because of endogenous plasminogen. LEFS requires an endogenous source of plasmin and is not responsive to activators. If standard assays using BFS as substrate are performed with nonactivated test solutions and with test solutions activated with streptokinase, the lytic activity observed in the former is a measure of "spontaneous" activator activity; whereas, the lytic activity of the latter is a measure of "total" activator activity. If standard assays using LEFS as substrate are performed with nonactivated test solutions and with test solutions activated with streptokinase, the lytic activity observed in the former is a measure of "spontaneous" plasmin; whereas, the lytic activity of the latter is a measure of "total" plasmin. The difference in activity measured by standard assays with BFS as substrate of the streptokinase activated test solutions and nonactivated test solutions is essentially the activity of proactivator. The difference in activity measured by standard assays with LEFS as substrate of streptokinase activated test solutions and nonactivated test

were used while in assays A and C nonactivated test solutions were utilized. The previously mentioned euglobulin dilutions were used because the fibrinolytic response was in the range where activity was proportional to euglobulin concentration for all eight samples tested. These activities were then converted to BF units in order that a quantitative comparison of specific activities could be made.

Any test solution resulting in optical density increase of .015 or less, which was considered within the error of the standard assay, was reported as having no activity. The results of the experiment are tabulated in Table 4. It was noted that total activator activity (assay D) was from about 100 to 200 times greater than total plasmin activity (assay B). Six of the eight euglobulins (samples 8 through 14) had no measurable preformed plasmin. The two euglobulins having measurable amounts of plasmin without previous streptokinase activation (assay A of samples 15 and 16) probably contained material from patients having one or more of the many reported clinical conditions (136) where fibrinolytic response is increased. Only one euglobulin had significant spontaneous activator activity (assay C of sample 16) though this activity was a small fraction of total activator activity.

In the experiment described above, eight euglobulin fractions prepared from plasma mixtures of hospital patients were assayed according to the scheme presented in Table 3. The experiment was performed in order to apply the assay scheme to a preparation commonly used in plasma fibrinolytic assays. The activities obtained are

Table 3

Total Assay of Fibrinolytic Components in Human Euglobulins

The Assay System

Assay	Substrate	Test Solution	Activity
A	LEFS	Nonactivated Euglobulin	Plasmin
B	LEFS	Streptokinase Activated Euglobulin	Plasmin and Plasminogen
C	BFS	Nonactivated Euglobulin	Plasmin and Activator
D	BFS	Streptokinase Activated Euglobulin	Plasmin, Plasminogen, Proactivator and Activator

Calculation of Individual Activities

Component	Calculation ¹
Plasmin	A
Plasminogen	B - A
Activator	C - A
Proactivator	$D - C + (B - A)^2$

¹Calculations performed with activities expressed in BF units
² $C + (B - A)$ is usually small compared to D and in most cases may be taken as zero.

tabulated in Table 4. It is evident from the results obtained that all four components may be detected by the assay system if they are present in the test solution. In the majority of the euglobulins tested, however, the two active components, plasmin and activator, were not present in the preformed state in measurable quantities.

It is considered that the assay system utilized and discussed in this experiment can be of significant value in the clinical study of diseases involving clot formation or dissolution. Many previous studies have indicated an abnormal fibrinolytic response in a variety of disease states. The majority of these studies fail to specify which of the fibrinolytic components cause the thrombolytic abnormality. It is evident that an increase in either spontaneous activator or plasmin could cause a high degree of lytic activity. A decrease in either proactivator or plasminogen could suppress the fibrinolytic mechanism. The assay system presented, using the two substrates BFS and LEFS, is a method which can be used to specifically measure plasmin, plasminogen, proactivator and activator. It is possible, therefore, to detect which of these entities is present in abnormal amounts in a clinical condition involving thrombolytic pathology. A more enlightened approach to understanding and treating abnormal thrombolytic conditions is possible when specific quantitative data is available on the components which comprise the fibrinolytic system.

Table 4

Fibrinolytic Components in Plasma Euglobulins

Sample	Specific Activity ¹ in Standard Assays			D	Specific Activity ¹ of Component			
	A	B	C		Plasmin	Plasmin- ogen	Activator	Pro- activator
9	0	1.75	0	253	0	1.75	0	251
10	0	1.25	0	176	0	1.25	0	174
11	0	1.17	0	246	0	1.17	0	245
12	0	1.29	0	200	0	1.29	0	200
13	0	1.78	.064	166	0	1.75	.064	164
14	0	1.12	.076	243	0	1.12	.076	241
15	.149	1.93	.164	230	.140	1.79	.024	228
16	.216	1.04	.292	216	.216	.82	.292	215

¹Reported in BF units

proactivator, activator, plasminogen and plasmin in euglobulin fractions of human plasma. This system provided an extremely simple and reproducible method for the study of the human fibrinolytic system.

VI. CONCLUSION

Because bovine fibrin suspensions contained large amounts of endogenous plasminogen, they served as excellent substrates for the assay of plasminogen activators. These activators transformed the endogenous plasminogen to plasmin which was detected by its ability to release acid-soluble proteolytic products.

Plasma activator and proactivator were quantitatively adsorbed by the fibrin suspensions while urokinase, the plasminogen activator in urine, was not. Rate studies indicated that plasma activator reacted stoichiometrically with endogenous plasminogen in the suspensions while urokinase reacted in a different manner. Plasma euglobulins, previously believed to contain the major portion of the fibrinolytic components of whole plasma, had from 39 percent to 79 percent of the whole plasma activator activity in eight samples tested after streptokinase activation.

Lysine solutions, because of their plasminogen solubilizing properties, effectively extracted the endogenous plasminogen from bovine fibrin suspensions. Lysine-extracted bovine fibrin suspensions, because of their lack of endogenous plasminogen, were effective substrates for the assay of plasmin.

With both the lysine-extracted and the unextracted bovine fibrin suspensions, a system of four assays was utilized to detect

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