THE ROLE OF A CONTAMINANT IN THROMBIN IN THE HUMAN PLASMIN ASSAY SYSTEM

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In recent years there has been increasing interest in the use of plasminogen as a potential therapeutic agent in the treatment of thrombotic disorders. Much work has been done on the isolation (1-5), mechanism of activation (6-9), characterization (10), and development of assay methods (11-13), for the components of the fibrinolytic system. Two assay methods are generally used. One measures the proteolytic activity of the active enzyme on a casein substrate; the other the lysis of a fibrin clot produced with a standard amount of bovine fibrinogen. Unfortunately bovine fibrinogen is reported to be contaminated with bovine plasminogen (14), and interpretation of assay results always requires examination of the role this contaminant could have played in determining the rate of clot lysis. A "human plasminogen" preparation contains two, at this time, inseparable substances—proactivator and proenzyme (plasminogen). The best known activator is streptokinase. Streptokinase converts proactivator to activator, which in turn converts the proenzyme plasminogen to the active enzyme plasmin. Plasmin can then be assayed by the proteolytic or fibrinolytic method. The rate of casein digestion is relatively low, but a tremendous amount of fibrin can be lysed by the enzyme in a very short time. The high fibrinolytic activity has been thought to be due to activation by the human activator of the bovine plasminogen contaminating the bovine fibrinogen substrate (14). Our calculations have revealed that the amount of bovine plasminogen that would have to be present as a contaminant to account for this activity is greater than the amount of bovine fibrinogen present in the assay system. In addition, there are reports of the great

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fibrinolytic activity of streptokinase-activated human plasmin, coupled with a low fibrinogenolytic activity, whereas bovine plasmin exhibits comparable fibrinolytic and fibrinogenolytic activity (15). Some authors have attributed the difference in fibrinolytic and fibrinogenolytic activity to a potentiation of human plasmin activation in the clotting process (16). These puzzling and contradictory reports in the literature on any measurement when clotting has occurred prompted our investigation of the role of thrombin in the plasmin assay systems. It was soon apparent that the thrombin preparation was a key factor in these anomalous results, and we are reporting here the observations which led to this conclusion. Moreover, we have isolated from bovine thrombin preparations the factor responsible for the confusion and have shown it to be similar in physical and enzymatic properties to bovine plasminogen.

Materials and Methods

Fibrinopeptide.—One ml. of normal oxalated human plasma was diluted to 10 ml. with water, 2 ml. of 0.1 M CaCl₂ was added, and the mixture clotted with 0.2 ml. (100 N.I.H. units) of thrombin. After 30 minutes the clot was spun out on a glass rod and the supernate placed in a 23/32 inch Visking sausage casing. The casing was immersed in 5 ml. of phosphate buffer (0.1 M, pH 7.4) in a 20 ml. vial and rocked at 4 ° for 24 hours. The dialysate should contain the same concentration of the dialyzable fibrinopeptide as the contents of the dialyzed bag.

Thrombin.—Parke, Davis and Co., Detroit, thrombin topical was used in all assays unless otherwise stated. A highly purified thrombin prepared by Dr. Walter H. Seegers was used in a single assay, as was a commercial Upjohn preparation. The commercial preparations were dissolved at a concentration of 500 units/ml. of saline. One mg. of the purified Seegers preparation was dissolved in 1 ml. of saline.

Bovine Fibrinogen.—(Armour and Company Laboratories, Chicago). A 1 per cent solution in veronal buffer was used in the fibrinolytic assays.

Veronal Buffer.—4.12 gm. of sodium barbital per liter adjusted to pH 7.4 with sodium hydroxide.

Streptokinase (Varidase.—Lederle Laboratories, Pearl River, New York).—100,000 units streptokinase and 25,000 units streptodornase were dissolved in 10 ml. of saline.

Bovine Plasminogen.—Bovine plasminogen was obtained through the courtesy of Dr. E. Loomis of Parke, Davis and Co.

Human Plasminogen.—Human plasminogen prepared by a modification (17) of the Kline method (18) was obtained from the Merck, Sharp and Dohme Research Laboratories, West Point, Pennsylvania. 35 mg. was dissolved in 95 ml. of water and adjusted to neutral pH by the rapid addition of 5 ml. of 2 M phosphate buffer.

Human Fibrinogen.—Human fibrinogen was prepared from a pool of oxalated normal human plasma by the method of Ware, Guest, and Seegers (19).

Casein.—A 1.8 per cent solution of casein in pH 7.4 phosphate buffer was prepared by the method of Mullertz (6), and stored frozen in small bottles.
Potato Starch.—Powdered Maine potato starch was freed of ultraviolet-adsorbing impurities by washing each pound with 1 liter of 0.01 M acetic acid at 50°, removing the acid by many distilled water washes, washing with phosphate buffer, water, alcohol, ether, and air drying.

Radioiodine.—Iodine-131 was obtained through the generosity of the Oak Ridge Laboratories of the Atomic Energy Commission. 0.5 mc. of the carrier-free isotope was used to label the proteolytic enhancing factor isolated from commercial thrombin.

Methods

Fibrinolytic Assay.—The method previously reported (2) has been used. In the determination of the effect of fibrinopeptide on fibrinolytic activity, 0.4 ml. of the fibrinopeptide dialysate was added to a mixture of 0.035 mg. of "human plasminogen," 1000 units of streptokinase (SK), 0.15 ml. of 1 per cent bovine plasminogen, and 0.15 ml. of veronal buffer. In assays to determine the effect of the amount of thrombin, the fibrinopeptide was replaced by 0.4 ml. of veronal buffer. In all assays the volume of thrombin solution used for clotting was 0.1 ml., and the amount of thrombin used was varied by dilutions with veronal buffer. In assays in which human fibrinogen was used as a substrate, 0.15 ml. was used. In determining the "human plasminogen"-lysis time curve of Fig. 5, 1000 units of streptokinase were placed in each tube and varying dilutions of plasminogen solution added for a 3 minute activation period prior to the addition of 0.15 ml. of bovine fibrinogen and sufficient veronal buffer to bring the volume to 0.9 ml. Clotting was accomplished with 50 units of thrombin.

Proteolytic Assay.—The proteolytic assay method was a previously reported variation (7) of the method of Mullertz (6). When thrombin was present in the assay system, it was added just prior to the addition of the final reactant, casein. Since activation of plasminogen preparations with streptokinase is very rapid, there is no effect of varying the order of addition of the reagents. In the determination of proteolytic activity in the presence of thrombin in which streptokinase concentration was varied, 3.0 ml. of casein solution was added to 3.0 ml. of a phosphate buffer solution containing 250 units of thrombin, 0.52 mg. of "human plasminogen," and 0 to 5000 units of streptokinase. The mixtures were incubated for 4 hours, and the results are reported as the γ of tyrosine liberated in this time (Fig. 1). The proteolytic enhancing activity of the starch electrophoresis eluates were determined similarly. Either 2 ml. of each eluate was added to 1 ml. of phosphate buffer, or 1 ml. of eluate was added to 2 ml. of buffer containing 0.35 mg. of "human plasminogen" and 150 units of streptokinase. After the addition of 3.0 ml. of casein 2 minute and 4 hour samples were assayed for tyrosine. Spontaneous proteolytic activities were also determined on the eluates by a similar assay replacing the 1 ml. of plasminogen and streptokinase by 1 ml. of phosphate buffer.

In the experiment in which proteolytic activity was measured in the absence and presence of bovine fibrinogen, the same amount of "human plasminogen" was used as above, and either 1.5 ml. of phosphate buffer or 1.5 ml. of 1 per cent fibrinogen was added. The "human plasminogen" was pre-activated with 0 to 3600 units of
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strepokinase/mg., and a 0.1 ml. portion of the activated mixture was used for fibrinolytic determination. (Fig. 4)

**Starch Electrophoresis.**—Starch electrophoresis of the thrombin preparation and of the bovine plasminogen was carried out for 17 hours at 4° on a 40 cm. starch block of 1 cm. thickness and 19 cm. width. A pH 7.6 phosphate buffer \( \frac{G}{2} = 0.1 \) was used with filter paper strips connecting the starch block to the electrode vessels. Approximately 50 ma. were drawn at a potential difference between the platinum electrodes of 400 volts. 5000 units of thrombin were dissolved in 3 ml. of water and dialyzed for 2 hours against a portion of the electrophoresis buffer. A 0.5 cm. section was cut out of the center of the starch block and the hole filled with a dry paste of the dialyzed thrombin solution and starch. The starch block was allowed to equilibrate for 1 hour with the buffer in the electrode vessels before the current was turned on. At the completion of electrophoresis the block was cut into sections of 1 cm. width and the protein eluted by transferring the section to a sintered glass funnel and stirring with 5 ml. of saline. The concentration of protein eluted from each centimeter section of the starch block was estimated by measuring the optical density at 275 m\( \mu \) of the eluate in 1 cm. cuvettes in a Beckman spectrophotometer. Other assays were then carried out on the eluate. Starch electrophoresis of 60 mg. of bovine plasminogen and of a mixture of 30 mg. of bovine plasminogen and the radiiodinated plasmin-enhancing factor from thrombin were carried out similarly. Radioactivity was measured in a \( \gamma \) well counter using a filter which diminished activity by a factor of 32.8.

**Clotting Time.**—A 0.1 ml. portion of the starch electrophoresis eluates was blown into a solution of 0.15 ml. bovine fibrinogen in 0.75 ml. of veronal buffer in a 13 x 100 mm. test tube, and the tube gently tilted until clotting occurred.

**Radioiodination of the Thrombin Contaminant.**—The starch electrophoresis eluate containing the proteolytic activity enhancing factor was lyophilized and iodinated with I\(^{131} \) according to the method of Pressman and Eisen (20).

RESULTS AND DISCUSSION

**Fibrinopeptide.**—If one is to accept the view of Bastian and Hill (16) that the process of clotting accelerated the activation of plasminogen with streptokinase, then some substance formed during the clotting process must be responsible for this acceleration. The peptide separated from fibrinogen by the action of thrombin might reasonably be expected to be that substance. When fibrinopeptide dialysate was added to the fibrinolytic assay system containing human plasminogen and streptokinase, and the mixture clotted with a \( \frac{1}{50} \) thrombin dilution (1 unit); a lysis time of 8 minutes was observed. In the absence of the fibrinopeptide the clot also lysed in 8 minutes. The presence of the additional fibrinopeptide therefore had no effect on the rate of lysis. However, if the same mixture was clotted with undiluted thrombin (50 units) a lysis time of 2.5 minutes was observed. Since the fibrinopeptide is obtained from the breakdown of fibrinogen and the fibrinogen content of the two assay
systems remains unchanged, it is difficult to see how more fibrinopeptide would be formed in the presence of excess thrombin or indeed how fibrinopeptide could be involved.

**Fibrinolytic Activity of Fibrinolytic Systems Clotted with Varying Concentrations of Thrombin.**—The discordant lytic activities observed above at two different thrombin levels suggested that thrombin concentration might have a determining role in fibrinolytic assay. Five different thrombin concentra-

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<th>Units of thrombin</th>
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<td>5</td>
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<td>2</td>
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<td>1</td>
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**TABLE II**

|  | Proteolytic Activity of Plasmin |
|  | 4 tyrosine                      |
| 4 hours |                             |
| (1) 250 units thrombin          | 225                           |
| (2) 0.52 mg. “human plasminogen” plus 1000 units SK | 304                           |
| (3) 250 units thrombin and 0.52 mg. “human plasminogen” and 1000 units SK | 1360                          |
| (4) 250 units thrombin and 0.52 mg. “human plasminogen” | 235                           |
| (5) 0.52 mg. human plasminogen | 10                            |
| (6) 1000 units SK               | 2                             |

Seegers' highly purified thrombin at concentrations of 0.1, 0.01, and 0.001 per cent were used to clot the same mixture of human plasminogen, streptokinase, and bovine fibrinogen. Lysis times of 9, 9, and 10 minutes respectively were observed. The purified thrombin clotted this mixture as rapidly as the Parke, Davis preparation yet it failed to produce the same rapid lysis. Since
Fig. 1. Increase in proteolytic activity of plasminogen and plasminogen plus thrombin in the presence of increasing amounts of streptokinase (SK). The results are expressed as the $\gamma$ of tyrosine liberated in 4 hours from a 0.9 per cent casein substrate by 0.52 mg. plasminogen in the presence and absence of 250 units of commercial bovine thrombin.
the high thrombin concentration was not responsible for the increase in lysis rate, then there must be some component in the commercial thrombin preparation which is not thrombin, and which can contribute to plasmin activity.

**Fibrinolytic Activity of Human Fibrinogen and Streptokinase Clotted with Thrombin.**—Human fibrinogen containing added streptokinase and clotted with 1 unit of thrombin required more than 2 hours for lysis. When clotted with 50 units of thrombin, lysis occurred in 15 minutes. Human fibrinogen prepared by the method of Ware, Guest, and Seegers under the action of streptokinase contains no fibrinogenolytic activity. It is therefore free of both proactivator and proenzyme together but it may contain one or the other. When 1 unit of thrombin is added for clotting no lysis occurs. However, 50 units of thrombin supplies sufficient quantity of one of the fibrinolytic components missing from the human fibrinogen for lysis to occur in 15 minutes. If human fibrinogen contains proactivator, then the proenzyme is supplied by the high concentration of thrombin. If the human fibrinogen contains proenzyme, then the high concentration of thrombin must supply proactivator. However, the latter possibility must be ruled out since the thrombin is of bovine origin and bovine proactivator is not converted to activator by streptokinase.

**Proteolytic Activity of Human Plasminogen and Streptokinase in the Presence of Thrombin.**—The tyrosine produced in 4 hours by the action of various enzyme and enzyme mixtures is reported in Table II. The sum of the tyrosine liberated by (a) thrombin alone, and by (b) human plasminogen together with streptokinase is far less than that liberated by (c) a mixture of thrombin, human plasminogen, and streptokinase. In this system which contained no fibrinogen and in which no clotting has occurred, there was a marked enhancement of the proteolytic activity of plasmin in the presence of thrombin. These results were of such great interest that the experiment reported in Fig. 1 was set up. Less than 30 units of streptokinase activated enough of the human proactivator to convert all of the human proenzyme to plasmin, and in the absence of thrombin a maximum plateau in the proteolytic activity was observed. Further additions of streptokinase could bring about no increase in proteolytic activity since all the proenzyme had already been converted to active plasmin. In the proteolytic assay to which thrombin had also been added, proteolytic activity increased as streptokinase concentration increased, and was still increasing at 5000 units of streptokinase, a level at which inhibition begins to appear in the absence of thrombin (21).

Since the human plasmin content of the proteolytic system remains constant after the addition of 30 units of streptokinase, there must be some other substance present which can combine with increasing amounts of streptokinase and bring about the increased activity observed in the presence of the thrombin preparation. In a previous report (7) it was shown that “human
Fig. 2. Properties of starch electrophoresis eluates of commercial bovine thrombin. Proteolytic activities are expressed as in Fig. 1. The clotting time is expressed as the seconds required for 0.1 ml. of eluate to clot 1 ml. of 0.15 per cent bovine fibrinogen in veronal buffer. The scale is inverted so that high activities appear at the top of the figure. Protein concentrations are expressed as the optical density at 275 mμ. 2 ml. of eluate were activated with 0.35 mg. of “human plasminogen” and 150 units of streptokinase.
plasminogen" contains a large excess of proactivator. On conversion to activator it can bring about the activation of plasminogen of human or bovine origin. If the thrombin preparation which is of bovine origin contains a large amount of bovine plasminogen as impurity, then the results of Fig. 1 could be anticipated. To confirm this explanation of the enhanced proteolytic activity of human plasmin in the presence of thrombin, electrophoretic investigation of the thrombin preparation was undertaken. It is to be noted that thrombin itself has some caseinolytic activity, but this is a property of the enzyme itself and is independent of any added streptokinase. At 1.4 units of streptokinase/mg, plasminogen, the proteolytic activity of (thrombin plus plasminogen plus streptokinase) is less than that of the sum of thrombin alone and (plasminogen plus streptokinase) alone. This is probably due to the presence of a small amount of plasmin inhibitor in the thrombin preparation.

Starch Electrophoresis of Thrombin Preparations.—Fig. 2 shows the results of the starch electrophoresis. The protein curve shows the presence of three peaks at 5, 8.5, and 12.5 cm. The thrombin preparation was placed on the block at the 7 cm. mark. The bulk of the protein moved toward the anode. The rapidly moving peak at the 12.5 cm. coincided with the peak in clotting activity. This must be the thrombin peak. At 8.5 cm. a peak was observed in proteolytic enhancing activity. This must be the bovine plasminogen peak. The small peak at 5 cm. of material moving toward the cathode is the small amount of plasmin inhibitor. The low peak at 12.5 cm. of eluate with spontaneous proteolytic activity (or activity in the absence of "human plasminogen" and streptokinase) is due to the caseinolytic activity of pure thrombin, and accounts for the activity observed in Fig. 1 when 0 units of streptokinase was added to the thrombin and plasminogen mixture. 0.1 ml. of the 9 cm. and 0.1 ml. of the 14 cm. eluate were used for clotting a fibrinolytic assay system, each containing the same amount of fibrinogen, "human plasminogen," and streptokinase. These eluates were chosen because each contained nearly the same units of thrombin as demonstrated by clotting time, but the 9 cm. material was rich in proteolytic enhancing factor while the 14 cm. one was practically devoid of it. The tube clotted with the 9 cm. material lysed in 5.5 minutes; that clotted with the 14 cm. material lysed in 10.5 minutes. Therefore, the proteolytic enhancing material is also a fibrinolytic enhancing material—both of which are activities associated with the active enzyme plasmin. All the enzymatic evidence points to the contaminant in thrombin as being identical with bovine plasminogen. A further electrophoretic investigation was undertaken to show their identity.

Electrophoresis of Radioiodinated Enhancing Factor.—Figs. 3 A and 3 B show the results of the starch electrophoresis of bovine plasminogen and of bovine plasminogen to which the radioiodinated plasmin-enhancing factor separated
Fig. 3. Starch electrophoresis of A, bovine plasminogen; and B, bovine plasminogen plus radiiodinated plasmin-enhancing factor separated from thrombin. Material was placed on the starch block at 7 cm. and under the influence of the electric field moved toward the anode. 1 ml. eluates of bovine plasminogen were assayed for proteolytic activity after activation with a streptokinase-activated “human plasminogen” preparation. A base line is shown in each figure for the proteolytic activity due to the “human plasmin.”
Fig. 4. Increase in enzyme activity of "human plasminogen" with increasing amounts of streptokinase measured on (A) casein, (B) casein plus bovine fibrinogen, (C) bovine fibrin. Proteolytic activities are reported in the previous figures. Lysis times are reported in minutes. The scale is inverted so that high enzyme activities appear at the top of the figure.
Fig. 5. Lysis time-concentration curve of "human plasminogen" activated with 1000 units of streptokinase.
from thrombin has been added. The bovine plasminogen was not electrophoretically homogeneous, and shows two bumps, as well as a peak, on the protein curve. (Fig. 3 A) The bulk of the protein scarcely moved in the electrophoretic field, but the bumps at 9 and 11 cm. suggest the presence of at least two other components. Since there is a sharp peak in proteolytic activity at 9 cm. the material responsible for the bump here on the protein curve is likely to be the bovine plasminogen. The low specific activity of bovine plasminogen is understandable in view of the large amount of inert protein which makes up most of the bovine plasminogen preparation. In Fig. 3 B, in which 5 mg. of iodinated plasmin-enhancing factor separated from thrombin was mixed with 30 mg. of bovine plasminogen before electrophoresis of the mixture, the proteolytic activity peak again appeared at 9 cm., and coincided exactly with the peak in radioactivity. Therefore, radioiodinated plasmin-enhancing factor from thrombin behaves electrophoretically exactly as does bovine plasminogen. The location of the proteolytic activity peak at 9 cm. in the electrophoretic curves of both the bovine plasminogen alone, and bovine plasminogen plus plasmin-enhancing factor makes it unlikely that the coincidence of the radioactivity and proteolytic activity peaks of Fig. 3 B is due to a complex formation between bovine plasminogen and iodinated plasmin-enhancing factor. Indeed, it is strong evidence that the two substances are identical.

Plasminogen Content of Fibrinolytic Assay Systems.—The experiment reported in Fig. 4 was set up to measure the proteolytic activity of the bovine plasminogen contaminant in a commercial bovine fibrinogen preparation. The same relative amounts of “human plasminogen” and fibrinogen were used in the proteolytic test as were used in the fibrinolytic test. The proteolytic activity of a mixture of “human plasminogen” and streptokinase on casein alone is shown on curve A, the proteolytic activity on casein containing bovine fibrinogen on curve B, and the fibrinolytic activity (fibrinogen clotted with 50 units of thrombin) on curve C. The proteolytic activity in the presence of bovine fibrinogen is only slightly higher than that on casein alone at low streptokinase concentrations and reaches a maximum value which is only twice as great as that of curve A at high streptokinase concentrations. This indicated that the proteolytic activity due to the bovine plasminogen contaminant of fibrinogen is just about equal in value to the proteolytic activity of the human plasmin alone. At 36 units of streptokinase the total (human plus bovine) plasmin activity is 400 μg tyrosine/4 hours. At 1000 units of streptokinase, it is 600. Proteolytic activity increased only 50 per cent; yet over that same streptokinase range the lysis time dropped from 15 minutes to 3.5 minutes. According to Fig. 5 this represents a 26-fold increase in plasmin fibrinolytic activity. Bovine fibrinogen appears to contain small amounts of bovine plasminogen in the proteolytic test, yet tremendous amounts are
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present in the fibrinolytic test. The fibrinolytic test contains the one additional reagent, thrombin, which is missing from the proteolytic test, and it is this which contains the bulk of the bovine plasminogen. In Fig. 1 the relative amount of thrombin to human plasminogen plus streptokinase is only one-third of the amount in the fibrinolytic test, and yet seven times as much fibrinolytic activity is observed in the presence of thrombin as in its absence at high streptokinase concentrations. At three times this thrombin concentration (the relative amounts in the fibrinolytic test) there would be a 21-fold increase in plasmin proteolytic activity. This is in good agreement with the 26-fold increase observed in fibrinolytic activity when 50 units of thrombin are used for clotting.

Reevaluation of Fibrinolytic Activities.—Interpretation of much of the work in the literature on the components of the fibrinolytic system is based on measurements of fibrinolytic activity. When this activity has been measured on systems containing human plasma fraction and streptokinase the role of the thrombin contaminant can be of prime significance. Until measurements can be repeated on fibrin substrates free of a bovine plasminogen contaminant in thrombin the claims from this laboratory (2) that plasmin is “primarily fibrinolytic rather than proteolytic when compared with trypsin” and that soy bean inhibitor fails to inhibit plasmin fibrinolytic activity must be withdrawn. A few papers (14–16) have seemed puzzling to us, and introduction of bovine plasminogen could account for the results which did not fit in with our general concept of the plasmin system.

Alternate interpretations could be advanced for many other papers appearing in the literature. It is to be hoped that investigators in this field will keep in mind that any experiment in which bovine thrombin has been used for clotting is an experiment which must be closely examined to see whether large amounts of bovine plasminogen fortuitously added could be responsible for the observed results.

SUMMARY

Many of the anomalous results obtained in the fibrinolytic assay of human plasmin systems were shown to be simply explained if bovine plasminogen had been introduced into the assay system on the addition of thrombin. Experimental investigation of the proteolytic and fibrinolytic activity of systems containing plasmin and thrombin showed that enzyme activity was influenced by the presence and quantity of thrombin. The quantity of bovine plasminogen present as a contaminant in bovine fibrinogen was shown to be responsible for only 1/28th of the observed enhanced activity. Thrombin in the amounts commonly used for clotting contained sufficient proenzyme to account for all this activity. A highly purified thrombin preparation obtained from another laboratory, and thrombin purified in this laboratory by starch
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Electrophoresis brought about no enhancement of activity. The material separated from thrombin by starch electrophoresis was shown to be enzymatically identical with bovine plasminogen and, on labelling with radioactive iodine, was shown to behave physically like bovine plasminogen. Several experiments reported in the literature were reinterpreted in the light of this observation.

REFERENCES


17. Data to be published.
21. Data to be published.