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Purification and Properties of a Plasminogen Activator from Pig Heart*

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EDMOND R. COLE‡ AND FEDOR W. BACHMANN§

From the Coagulation Laboratories, Section of Hematology, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612

An improved procedure is described for the purification of plasminogen activator from pig heart. The initial purification steps were similar to those described previously (Bachmann, F., Fletcher, A. P., Alkjaersig, N., and Sherry, S. (1964) *Biochemistry* 3, 1578–1585). Use of a novel extraction medium containing EDTA, cysteine, and 2,3-dimercaptopropanol-1 facilitated the removal of large amounts of inert proteins prior to gel filtration on Bio-Gel P-150. The final product had a specific activity of 120,000 to 160,000 CTA units/mg of protein (CTA, Committee on Thrombolytic Agents of the National Heart Institute). Total purification over pig heart was 25,000 to 30,000-fold, average recovery compared to the initial extract was 6 to 8%. Polyacrylamide gel electrophoresis revealed a major and two minor components. The molecular weight of the activator determined by gel filtration was 51,500 ± 3,400 for the major activity component and 48,000 for a minor component which was partially separated from the major peak in eight of nine chromatography runs. A γ-globulin fraction of antiserum against purified activator neutralized the biological activity of the activator on fibrin plates. Immunoelectrophoresis of gel-filtered activator revealed only one anodic component.

The fibrinolytic system is the last line of defense against the permanent occlusion of blood vessels by fibrin. The endothelial cell contains an activator of plasminogen (1–3) and it is believed that this activator is released during the formation of a thrombus. Activation of plasminogen then results in the formation of a proteolytic enzyme capable of lysing the fibrin network into soluble fragments. Many attempts have been made to isolate and purify the activator of plasminogen, known as tissue activator. Because of the close association of tissue activator with particulate cellular material and its apparent insolubility at neutral pH, little success at purifying tissue activator was made until Astrup and Stage (4) found 1 M solutions of KSCN capable of solubilizing the activator. The KSCN extracts could be further purified by acid precipitation, and quantitative methods of assay were developed (5). Subsequently, concentrated urea solutions (6) and acidic buffers (7) were found to extract tissue activator from pig heart. Although all these agents solubilized not only tissue activator but other cellular components as well, they have served well to produce initial extracts for studies on the purification of tissue activator of plasminogen (7–9). Kok and Astrup (8), as well as Bachmann *et al.* (7), using different extraction and purification procedures, achieved preparations with specific activities in the range of 10,000 to 25,000 CTA[1] units/mg. Both of these products showed electrophoretic heterogeneity. More recently, Rickli and Zaugg (9) reported on a purification procedure resulting in activator (pig heart tissue activator) with a specific activity of 39,000 CTA units/mg. Disc electrophoresis in polyacrylamide gels revealed a single band by visual inspection, but four additional components in trace amounts by densitometric scanning of the stained gel.

In all previously reported methods, gel filtration was a useful step in the purification of tissue activator and resulted in 20- to 100-fold increases in specific activity. However, such preparations contain inactive proteins of a molecular size similar to that of tissue activator, and it is doubtful that repetitive use of methods separating proteins according to size will yield further significant increases in specific activity.

This paper describes the purification of tissue activator to specific activities of 120,000 to 160,000 CTA units/mg, corresponding to a 25,000- to 30,000-fold purification compared to the initial pig heart suspension. This has been achieved in large part through the use of a novel extraction system employing a chelating agent and sulfhydryl compounds which allows separation of activator from most of the other cellular proteins having similar molecular size and physicochemical properties.

MATERIALS AND METHODS

Fresh frozen pig hearts were obtained from Pel-Freeze Biologicals, Inc., Rogers, Ark., and maintained at −70° until used. Assay of activator was performed by human plasma clot lysis, bovine fibrin plate lysis, and human fibrin plate lysis. The plasma pool used for the human clot lysis assay (7) was obtained from the blood of two volunteers drawn in citrate/phosphate/dextrose blood donor packs. Aliquots of 2 ml of plasma were frozen at −70°. The assay was performed by placing 0.2 ml of thawed plasma in a glass tube (10 × 75 mm) in a 37° water bath. Tissue activator preparations were diluted in pH 7.35 Michaelis buffer, ionic strength 0.15. One-tenth milliliter of activator, immediately followed by 0.1 ml of Parke, Davis and Co. bovine thrombin, 20 NIH units/ml, were added to the tube. The time from addition of the tissue activator preparation to

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‡ To whom reprint requests should be addressed.

§ Present address, Office of European Research, Schering Corp. U.S.A., Toepferstrasse 5, 6004 Lucerne, Switzerland.

[1] The abbreviations used are: CTA, Committee on Thrombolytic Agents of the National Heart Institute.

complete lysis of the clot was taken as the clot lysis time. Standard curves were obtained using human urokinase,[2] and all results are expressed in CTA units. The reciprocal of the lysis time plotted against urokinase concentration, gave a standard curve usable over the range of 6 to 30 min at urokinase concentrations of 200 to 2,000 CTA units/ml. Reproducibility of the human clot lysis assay was ±5%.

Bovine fibrinogen (Fraction I, 65% clottable) was obtained from Pentex, Kankakee, Ill., and human fibrinogen[2] (90% clottable) from Cutter Laboratories, Berkeley, Calif. For human and bovine fibrin plate assays, the method of Mullertz (10) as modified by Alkjaersig *et al.* (11) was used. Tissue activator preparations were diluted in 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2, and applied to fibrin plates as 20-μl aliquots to yield lysis zones between 150 to 300 mm^2. The assays were standardized with human urokinase diluted in the same buffer. An aliquot of 20 μl of a solution of 2.5 CTA units of urokinase/ml typically produced lysis zones of 250 mm^2. Reproducibility of bovine fibrin plate and human fibrin plate assays were ±12% and 11%, respectively. In some experiments described, dilutions of urokinase and tissue activator were made in the pH 4.2 acetate:NaCl buffer containing 0.2% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, Mo.). In these experiments the clot lysis time was also measured by dilution of urokinase and tissue activator in Michaelis buffer containing 0.2% albumin. Nonspecific proteolytic activity was measured on fibrin plates heated to 80° for 30 min to inactivate plasminogen.

Disc polyacrylamide gel electrophoresis was performed by the method of Neville (12) using a stacking gel at pH 4.0 and a running gel at pH 2.7. Tissue activator preparations were in 0.01 M potassium acetate, 0.005 M NaCl, pH 4.2, to which a small amount of glycerol was added. Basic fuchsin was used as the tracking dye, but was run separately on another gel at the same time. Electrophoresis was carried out toward the cathode at 0.75 mA/tube while stacking and 1.5 mA/tube thereafter. Gels were stained overnight with Coomassie brilliant blue and destained by diffusion as described by Weber and Osborn (13). The stained gels were photographed as described by Oliver and Chalkley (14). To detect tissue activator activity on unstained gels, the gels were sliced into 2-mm segments with a gel slicer. Each slice was dipped into 0.1 M sodium borate buffer, pH 8.0, for 5 s and placed on a fibrin plate which was then incubated at 37°. The lysis zone was calculated as the difference between the product of the diameters of the total lysis zone and of the gel slice.

Protein determinations were by the method of Lowry *et al.* (15) using human crystallized albumin (Dade, Miami, Fla.) as a protein standard. Chromatography eluates were continuously monitored by an LKB Uvicord II detector and recorder unit at 280 nm, and absorbance of each fraction was also measured at 280 nm in a Beckman DU spectrophotometer.

Mixtures of 0.05 M Tris/HCl buffer, pH 7.4, 0.01 M EDTA, freshly prepared 0.05 M cysteine hydrochloride, and 0.03 M 2,3-dimercaptopropanol-1 in the ratio of 70:10:10:0.1 volumes, respectively, were prepared immediately before use and the pH adjusted to 7.4. Potassium acetate buffers were prepared from glacial acetic acid and the pH adjusted with KOH. Sodium chloride was added prior to diluting to volume.

The β-naphthylamides of L-lysine, L-arginine, and L-histidine were products of Schwarz/Mann, Orangeburg, N. Y. Amidase activity of tissue activator preparations was determined by the method of Blackwood and Mandl (16). Bio-Gel P-150 (100 to 200 mesh) was a product of Bio-Rad Laboratories, Richmond, Calif., and Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Molecular weight marker proteins were products of Schwarz/Mann and Pharmacia Fine Chemicals.

Molecular weight estimation of tissue activator was performed by gel filtration (17), using Bio-Gel P-150 in a glass column of 1.5 cm diameter, packed to a height of 56 cm. For equilibration and column elution, 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2, was used. Marker proteins were dissolved in the same buffer, 2 mg/ml, and centrifuged to remove small amounts of insoluble material. A sample volume of 2 ml was applied and the column developed at a flow rate of 11.5 ml/h under a hydrostatic head of 16 cm of H_2O. Fractions of 1.5 to 2.0 ml were collected and subjected to Lowry protein assay for marker proteins or for activator activity on bovine fibrin plates. Molecular weight also was estimated from elution volumes of tissue activator and marker proteins subjected to gel filtration on large Bio-Gel P-150 columns used in the preparative method of activator purification.

Activator antiserum was produced in rabbits by injecting intramuscularly 0.5 mg of chromatographed tissue activator (> 100,000 CTA units/mg) in complete Freund's adjuvant. A booster injection was given 1 month and 5 months after the initial injection, and 5 days later blood was obtained by heart puncture. Serum was harvested after spontaneous clotting and incubation for 18 h at 37°, and crude γ-globulin was prepared by 33% ammonium sulfate precipitation. A γ-globulin fraction was similarly prepared from serum obtained from a nonimmunized rabbit. After overnight dialysis of the γ-globulin preparations against cold 0.9% NaCl solution, the preparations were adjusted with saline solution to the same protein concentration, 16.6 mg/ml.

The quenching effect of activator antiserum γ-globulin on activator activity was demonstrated on bovine fibrin plates by incorporation into the plates the γ-globulin from nonimmunized and immunized rabbits at final dilutions of 1:1000, 1:5000, 1:10,000, 1:15,000, 1:20,000, and 1:25,000. Serial dilutions of activator (1.0 to 14.5 CTA units/ml) were applied to control plates and plates containing γ-globulin, and lysis areas were measured after 16 h at 37°.

Immunoelectrophoresis was performed at pH 8.6 in 0.05 M sodium barbital, 0.035% disodium EDTA using the Pol-E-Film system of Pfizer Diagnostics (Clifton, N. J.). Electrophoresis was conducted for 45 min at 90 V.

Enzyme Purification

Step 1: Acetone Drying—Four to five kilograms of partially thawed pig heart were ground in an electric meat grinder. One-kilogram portions were suspended in 4 liters of −20° acetone in a 6-liter Waring Blendor and further homogenized. Pieces of dry ice were added to maintain low temperature. Filtrations were performed in Buchner funnels using Whatman No. 541 paper and suction. The filter cake was then added back to the Waring Blendor and the homogenizing process was repeated four times. The final filter cake was fragmented and spread on large sheets of filter paper to dry at room temperature. The fine tan-colored powder weighed 600 to 700 g, or about 15% of the weight of wet tissue.

Step 2: Extraction by Acetate Buffer—For each 100 g of acetone-dried pig heart powder, 800 ml of 0.3 M potassium acetate, pH 4.2, was added and the suspension was stirred continuously for 6 h at 5°. The supernatant was recovered by centrifugation in the cold at 5,000 × *g* for 30 min, and the sediment re-extracted with 400 ml of 0.3 M potassium acetate, pH 4.2, for at least 3 h, again recovering the supernatant by centrifugation.

Step 3: First Ammonium Sulfate Precipitation—The combined supernatant solutions were continuously stirred while 300 g of ammonium sulfate/liter of extract were added over a 4-h period to give 50% saturation at 2°. After overnight settling, the settled precipitate was centrifuged and worked into a smooth paste and dispersed in 300 ml of cold distilled water/100 g of original dried product. The pH was adjusted to pH 4.2 with 1 M acetic acid and the suspension stirred for 2 h and centrifuged. The sediment was re-extracted overnight with 150 ml of 0.1 M potassium acetate, pH 4.2. Following centrifugation, both eluates were combined.

Step 4: Second Ammonium Sulfate Precipitation—The eluates were adjusted to pH 8.2 with solid Tris and finely powdered $(NH_4)_2SO_4$ was added slowly with stirring (200 g/liter, 35% saturation at 2°). One and one-half hours later, the solution was centrifuged and the precipitate, dissolved in 75 ml of 0.05 M acetic acid, was dialyzed overnight against two changes of 2.5 liters of cold distilled water to a resistance greater than 1,000 ohms.

Step 5: Zn^{2+} Precipitation at Low Ionic Strength—The dialyzed preparation was adjusted to pH 4.2 with acetic acid and the sample was cleared by centrifugation. The pH of the supernatant was adjusted to 6.0 with 1 N NaOH and a solution of 10 mM zinc acetate was added slowly to give a final Zn^{2+} concentration of 0.3 mM. The pH was adjusted to 6.5 with 0.1 M sodium barbital and stirred for 15 min. After settling for 1 h, the precipitate was recovered by centrifugation, dispersed into 20 ml of 0.5 M acetic acid, and the pH of the solution adjusted to 4.2, the solution was stirred until the solid dissolved and then lyophilized. Six hundred to seven hundred grams of acetone-dried pig heart yielded 2 to 2.5 g of lyophilized Step 5 product.

Step 6: Fractionation in Presence of EDTA and Sulfhydryl Reagents—One gram of lyophilized Step 5 activator was suspended in 180 ml of a 70:10:10:0.1 mixture of Tris:EDTA:cysteine:2,3-mercapto-

[2] We are indebted to Abbott Laboratories, North Chicago, Ill., for the gift of urokinase; and to Dr. A. R. Pappenhagen, of Cutter Laboratories, Berkeley, Calif., for the gift of human fibrinogen.

propanol-1, pH 7.4, and homogenized with a tissue homogenizer. The pH was adjusted to 7.4 with 0.1 M NaOH and the suspension was stirred for 20 min at room temperature. It was then centrifuged at 9,000 × *g* for 30 min at 5° and the supernatant was decanted. The residue was then taken up in 180 ml of 70:20 Tris:water, homogenized, and the pH was adjusted to 7.4. After stirring for 20 min at room temperature, the suspension was again centrifuged at 9,000 × *g* for 30 min and the supernatant was decanted. The residue was taken up to 20 ml of 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2, the pH of the suspension was adjusted to 4.2 and it was stirred for 30 min at room temperature and centrifuged at 5° at 13,500 × *g* for 30 min. The supernatant, which contained the bulk of the tissue activator activity, was chromatographed on Bio-Gel P-150.

Step 7: Gel Filtration on Bio-Gel P-150 – A glass column (2.5 × 100 cm) was packed to a height of 90 cm with Bio-Gel P-150 and equilibrated with 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2, at 5°. The tissue activator preparation (Fraction 6) was layered over the top of the gel and allowed to enter the gel. Then the column was developed with the 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2 buffer, at a flow rate of approximately 10 ml/h with a hydrostatic pressure of 60 cm of H_2O. Four-milliliter fractions were collected by drop counting.

Step 8: Acetone Precipitation of Column Fractions – Active column fractions were pooled and dialyzed overnight in the cold against distilled water. The pH was adjusted to 7.0 by addition of 0.1 M NaOH and −20° acetone was slowly added with continuous stirring to a final concentration of 33% while the temperature of the pool/acetone mixture was decreased from −5–−8°. The precipitate was recovered by centrifugation at −10° (13,500 × *g* for 30 min) and dissolved in 2 ml of 0.01 M potassium acetate, 0.005 M NaCl, pH 4.2. After assay for tissue activator activity and protein, an equal volume of glycerol was added. These solutions were then maintained at −20°.

RESULTS

Sephadex G-200 Gel Filtration of Step 5 Material – Step 5 material, 250 mg, was suspended in 10 ml of 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2, homogenized and stirred for 20 min at room temperature, and centrifuged at 9,000 × *g* for 30 min at 5°. Gel filtration of the supernatant on Sephadex G-200 revealed a chromatogram (Fig. 1) similar to that previously reported (7). Activator was eluted on the descending side of a large protein peak which appeared near the void volume. Recovery of activity, when measured by the human clot lysis assay, was 70%, and peak activity fractions had a specific activity of 5,500 CTA units/mg.

Extraction of Step 5 Material in Presence of EDTA and Sulfhydryl Compounds – The use of EDTA and sulfhydryl compounds for the fractionation of tissue activator arose as a consequence of studies to determine whether various synthetic esters and amides could be used to follow the degree of purification of tissue activator preparations. Hydrolysis of the β-naphthylamides of lysine, arginine, and histidine occurred in the presence of Step 5 supernatant, with L-lysine-β-naphthylamide the best substrate in terms of β-naphthylamide release. Hydrolysis of L-lysine-β-naphthylamide was two to three times increased in the presence of incubation systems containing EDTA and sulfhydryl compounds. These studies led to the fortuitous observation that elution of Step 5 material with Tris:EDTA:cysteine:2,3-mercaptopropanol-1 resulted in the dissociation of the amidase from bovine fibrin plate and human clot lysis activities. About 90% of the amidase activity and half of the protein were found in the supernatant, but most of the bovine fibrin plate and human clot lysis activities remained in the residue.

Table I illustrates the improved extraction procedure. The initial extraction of Step 5 material was performed in Tris:EDTA:cysteine:2,3-mercaptopropanol-1, pH 7.4 (Fraction a), prior to dissolution of the residue (Fraction b) in 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2. Analysis of this supernatant Fraction c revealed that it contained only 5.5% of the original protein, but 95% of human clot lysis and 69% of bovine fibrin plate activities, yielding specific activities of 3500 and 850 units/mg, respectively, or 8 to 9 times those achieved by acid/acetate extraction alone. Gel filtration of Fraction c on the same Sephadex G-200 column (1.5 × 84 cm) used in the previous experiment demonstrated that the Tris:EDTA:cysteine:2,3-mercaptopropanol-1 fractionation procedure resulted in a significant reduction of the first major protein peak seen in Fig. 1, and of the proteins associated with the activator activity peak (Fig. 2). The gel filtration step yielded fractions with specific activities of 34,000 to 36,000 CTA units/mg by human clot lysis assay, but produced no further increases of the human clot lysis/bovine fibrin plate ratio. Addi-

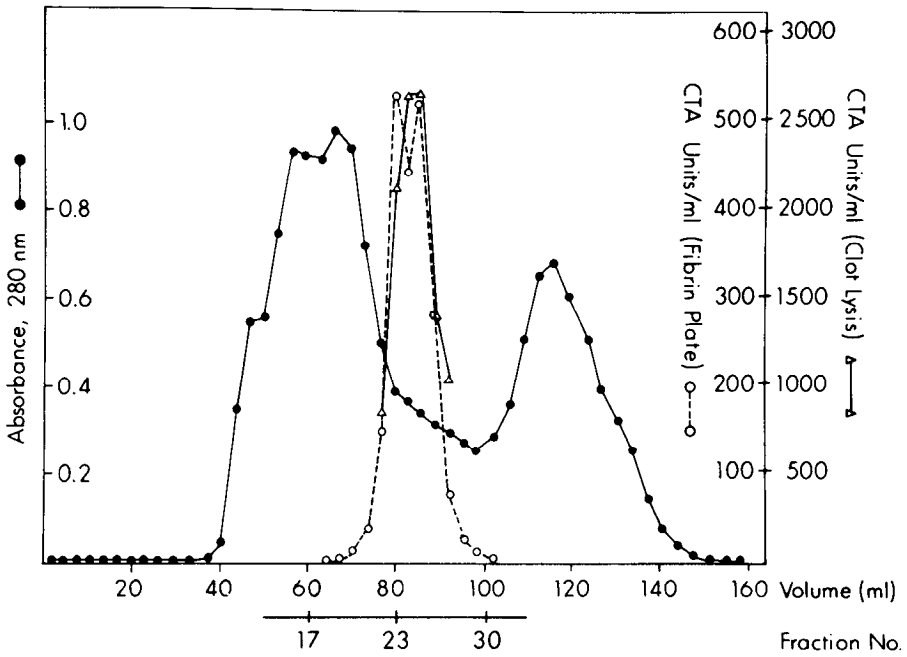

FIG. 1. Gel filtration of 7 ml of acid/acetate-extracted Step 5 material on a column (1.5 × 84 cm) of Sephadex G-200. Protein (102 mg), representing 175 mg of Step 5 material was used. Elution was performed with 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2. The activity of the eluate was determined by bovine fibrin plate and human clot lysis assays.

tional gel filtration experiments with Fraction c on Bio-Gel P-150 columns of the same size revealed a shift of contaminant proteins to lower elution volumes, reduced protein levels under the activity peak further to 30 to 40 μg/ml, and increased the specific activity of activator to 50,000 units/mg.

Optimal Conditions for Preparative Extraction of Activator Activity – Fig. 3 outlines the preparative extraction procedure adopted after many experiments and Table II compares the activity assays of individual fractions. Suspension of tissue activator in Tris:EDTA:cysteine:2,3-mercaptopropanol-1 always led to higher activities when measured by human clot lysis as compared to bovine and human fibrin plates (Fraction 1). Little activity was found in the supernatant (Fraction 2), although about 50% of the protein was removed. Resuspension of the sediment in Tris:H_2O in the ratio 70:20 (Fraction 3) and centrifugation led to further small loss of protein in the supernatant (Fraction 4), but no appreciable loss in activity. The use of Tris:EDTA:cysteine:2,3-mercaptopropanol-1 in the second extraction gave essentially the same results. However, Tris:H_2O was favored in the extraction because tissue activator recovery in subsequent extracts was greater. Suspension of the residue in 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2 (Fraction 5), reproducibly led to an apparent large loss in human clot lysis activity of tissue activator. This phenomenon was not observed when Fraction 5 was assayed by the bovine fibrin plate method; using the human fibrin plate assay the effect was intermediate. However, on separation of the suspension, there was a consistent apparent increase of total human clot lysis activity and of the human clot lysis/bovine fibrin plate ratio in the supernatant (Fraction 6), while the activity ratio of the residue (Fraction 7) was low. Re-extraction of Fraction 7 in acetate:NaCl, pH 4.2, and centrifugation yielded soluble Fraction 8 which contained about 20 to 25% of the total activity of Fraction 6 with similar specific activities and activity ratios.

Despite discrepant assay values, overall recoveries and specific activity increases were essentially the same for all three assay methods. The recovery of tissue activator activity in Fraction 6 was 45, 51, and 51% for bovine fibrin plate, human fibrin plate, and human clot lysis, while the specific activity increases were 36, 41, and 41 times, respectively. The mean total protein in Fraction 6 in nine runs represented 2.1% of the protein of Fraction 1 (range 1.2 to 3.2%). The mean specific human clot lysis activity of Fraction 6 was 6,140 CTA units/mg of protein (range 4,000 to 8,650 CTA units/mg).

There was apparent greater recovery of activator in Fraction 6 when 0.01 M potassium acetate, 0.005 M NaCl, pH 4.2, was used to prepare Fraction 5 suspension. However, gel filtration of Fraction 6 prepared with a low ionic strength buffer resulted in considerable spreading of the activator activity, especially on the descending side of the activity peak. This resulted in column fractions having specific activities in the range of 40,000 to 60,000 units/mg (human clot lysis assay), considerably lower than those achieved when higher ionic strength buffers were used. This relationship between buffer ionic strength and separation of activator from inert proteins by gel filtration has been noted previously (7, 9).

Assay Variations – Because of the discrepancy between bo-

TABLE I

Improved extraction procedure, using both EDTA-sulfhydryl compounds and acid/acetate

Fraction a represents 500 mg of Step 5 activator suspended in 90 ml of Tris:EDTA:cysteine:2,3-mercaptopropanol·1. Fraction b is the supernatant after centrifugation of Fraction a. The residue, suspended in 20 ml of 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2, was then separated into the supernatant (Fraction c) and residue (Fraction d) fractions.

Fraction	Total protein	Bovine fibrin plate			Human clot lysis			HCL/BFP[a] ratio
	mg	*U/ml*	*U/mg*	*Total U*	*U/ml*	*U/mg*	*Total U*	
a	510	380	64	34,400	1,150	190	103,500	3.0
b	221	15	6	1,300				
c	28	1190	850	23,800	4,900	3,500	98,000	4.2
d	220	300	28	6,000				

[a] HCL, human clot lysis, BFP, bovine fibrin plate; *U*, units.

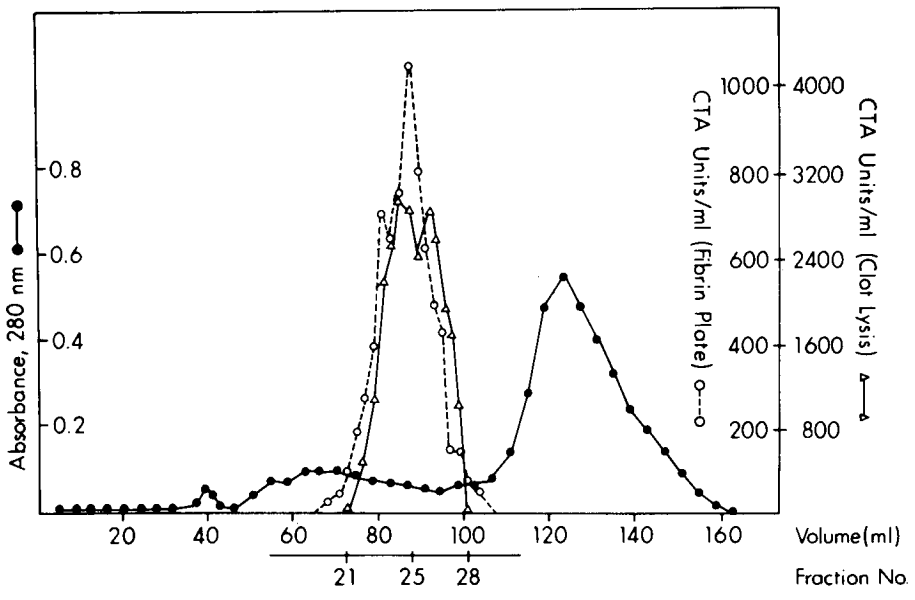

FIG. 2. Gel filtration of 11.5 ml of Step 5 material after extraction with EDTA/sulfhydryl compounds and acid/acetate (Fraction c of Table I) on a column (1.5 × 84 cm) of Sephadex G-200. Protein (16.1 mg), representing 283 mg of Step 5 material, was used. Elution was performed with 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2. The activity of the eluate was determined by bovine fibrin plate and human clot lysis assays.

vine fibrin plate, human fibrin plate, and human clot lysis assay values, specific activities and activity ratios were determined at all stages of the purification process. These results are summarized in Table III. Because clot lysis time assays require tissue activator concentrations approximately 100 times greater than those required for fibrin plate assays, the former could not be determined on fractions prior to the Zn^{2+} precipitation. Acetone-dried pig heart, suspended in 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2, yielded the same activity data by bovine and human fibrin plate assays. However, beyond this step through the 35% ammonium sulfate precipitation stage, human fibrin plate/bovine fibrin plate activity ratios were approximately 2. Although 84% of the human fibrin plate activity of Step 1 material was recovered in Step 2 material, only 38% was recovered when the activity was measured by the bovine fibrin plate assay. In the subsequent four purification steps, the recovery data as measured by both assays were comparable, resulting in a yield of 28 to 29% from Step 2 to Step 5.

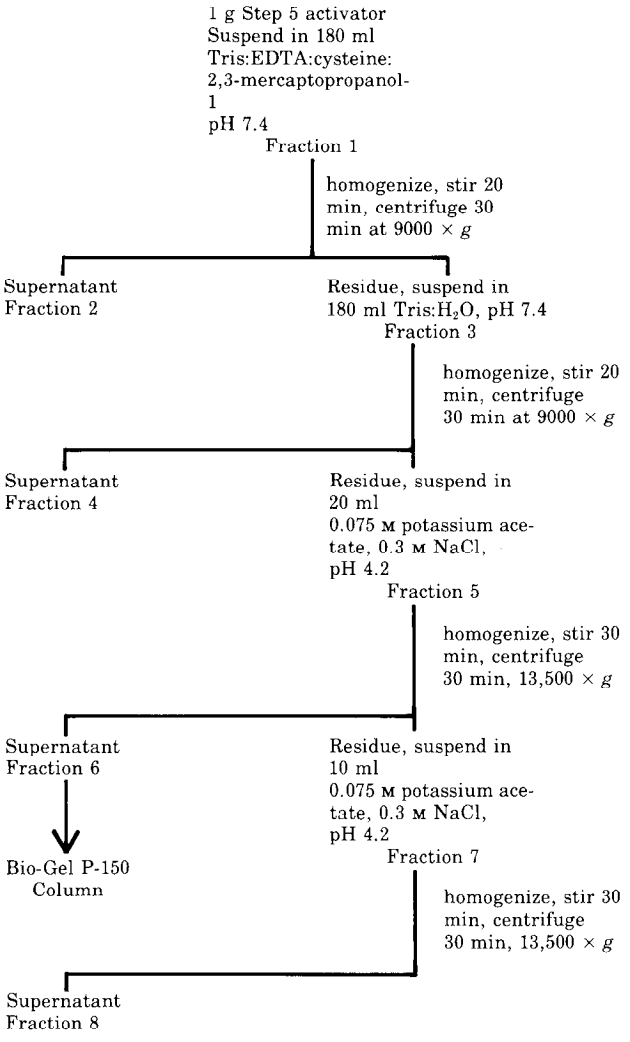

FIG. 3. Preparative fractionation scheme employing EDTA/sulfhydryl and acid/acetate extractions.

Activity ratios of activator in purification steps beyond Step 5 were dependent on the medium in which the preparation was suspended. As seen in Tables I and II, human clot lysis/bovine fibrin plates and human fibrin plate/bovine fibrin plate ratios were near 3 and 2, respectively, when Step 5 material was suspended in Tris:EDTA:cysteine:2,3-mercaptopropanol-1. However, when the same material was suspended in acid/acetate buffer, pH 4.2, the ratios were approximately 1.5 (Table I). Suppression of human clot lysis activities was seen at all stages where acid-soluble activator was in contact with acid-insoluble material. After removal of acid-insoluble material by centrifugation, a significant increase of total activity was regularly found in the supernatant, as demonstrated in Fraction 6, Table II. Thus, it appears that an inhibitor affecting the assays was present in the acid-insoluble fraction. The same phenomenon was observed to a lesser degree for the human fibrin plate assay in the nine different purification runs.

Preparative Gel Filtration of Fraction 6—Fig. 4 shows the chromatogram of a tissue activator concentrate prepared by the method shown in Fig. 3. The amount of protein applied in nine gel filtration experiments was 15.8 to 29.7 mg, equivalent to an average of 930 mg of Zn^{2+}-precipitated tissue activator. For rapid screening of column fractions for tissue activator activity, undiluted samples were applied to bovine fibrin plates. The most active fractions gave lysis areas within 1 h of incubation at 37°. Active fractions were assayed by the human clot lysis method and applied as four 20-μl spots, diluted to give lysis zones of 150 to 300 mm^2, on bovine and human fibrin plates. Tissue activator activity consistently appeared at the same elution volumes and was not associated with a well defined 280 nm absorbance. As seen in Fig. 4, there appear to be two incompletely separated activity peaks. Initially, this was attributed to assay errors, but it is significant that a double activity peak was seen in eight of nine chromatograms, although the relative activities of the two peaks varied from experiment to experiment.

TABLE II

Comparison of bovine fibrin plate, human fibrin plate, and human clot lysis assays of activator fractions obtained by preparative extraction procedure

Fraction	Total protein	Bovine fibrin plate			Human fibrin plate			Human clot lysis			Ratio[a]	
											HCL/BFP	HFP/BFP
	mg	*U/ml*	*U/mg*	*Total U*	*U/ml*	*U/mg*	*Total U*	*U/ml*	*U/mg*	*Total U*		
1	1,040	390	67	70,600	640	110	115,200	1,100	190	198,000	2.8	1.6
2	515	12	5	2,040	25	9	4,200					2.0
3	445	495	236	89,100	740	350	133,700	1,100	524	198,000	2.2	1.5
4	76	0	0	0	9	21	1,560					
5	265	4,380	332	87,600	4,800	370	97,600	3,120	237	62,500	0.7	1.1
6	13	1,730	2,480	32,100	3,200	3,080	59,200	5,400	7,710	99,900	3.1	1.8
7	245	1,330	54	13,400	1,380	57	13,800	550	23	5,500	0.4	1.0
8	3	840	2,270	7,140	1,370	3,720	11,700	2,700	7,300	25,600	3.2	1.6

[a] HCL, human clot lysis; BFP, bovine fibrin plate; HFP, human fibrin plate; *U*, units.

TABLE III

Initial enzyme purification steps

Step	Product	Human fibrin plate assay			Bovine fibrin plate assay			HFP/BFP[a]
		CTA U/mg	Total units	Step yield	CTA U/mg	Total units	Step yield	
				%			%	
1	Acetone powder[b]	5	1,934,400		5	1,999,100		1.0
2	pH 4.2 Acetate extract	24	1,632,200	84	11	752,200	38	2.2
3	50% $(NH_4)_2SO_4$ ppt.	43	1,247,600	76	18	522,100	69	2.4
4	35% $(NH_4)_2SO_4$ ppt.	65	1,008,200	81	31	501,400	96	2.0
5	Zn^{2+} ppt., dialyzed, lyophilized[c]	186	462,700	46	89	220,600	44	2.1
	% yield, Step 1 to Step 5			24			11	
	% yield, Step 2 to Step 5			28			29	

[a] HFP, human fibrin plate; BFP, bovine fibrin plate; U, units.
[b] Suspended in pH 4.2 potassium acetate, 0.3 M.
[c] Suspended in pH 7.4 Tris:EDTA:cysteine:2,3-mercaptopropanol-1.

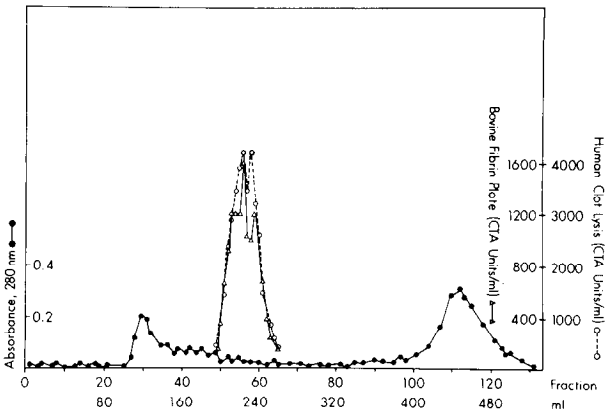

FIG. 4. Gel filtration of 17.6 ml of activator fraction obtained by extraction with EDTA/sulfhydryl compounds and acid/acetate by the preparative fractionation scheme (*Fraction 6* of Fig. 3) on a column (2.5 × 90 cm) of Bio-Gel P-150. Representing 930 mg of Step 5 material, 22.2 mg of protein was used. Elution was performed with 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2. The activity of the eluate was determined by bovine fibrin plate and human clot lysis. Activity was also measured by human fibrin plate assays (not shown).

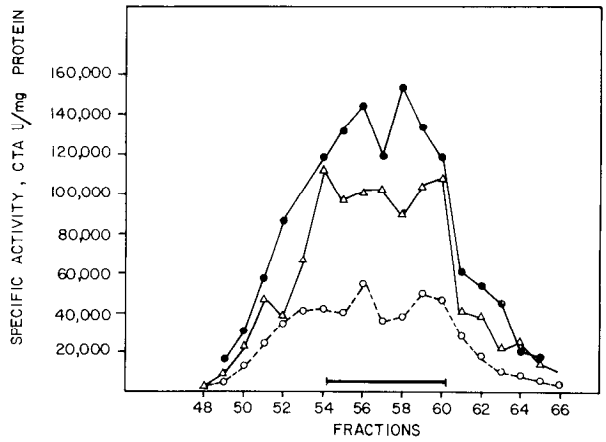

FIG. 5. Specific activity of activator fractions obtained by Bio-Gel P-150 gel filtration experiment shown in Fig. 4. (human clot lysis, ●——●; human fibrin plate, △——△; bovine fibrin plate, ○- - -○). The *graph bar* indicates the fractions pooled for acetone precipitation and subsequent polyacrylamide gel electrophoresis and immunoelectrophoresis.

Fig. 5 depicts the specific activities of Bio-Gel P-150 column fractions as determined by human clot lysis, bovine fibrin plate, and human fibrin plate assays. The specific activities tended to be rather constant in the center portion of the activity peak, suggesting some degree of homogeneity. However, the reduction in specific activities on the ascending and descending slopes of the peak also suggested contaminants. Fractions 54 to 60 inclusive showed a mean specific activity of 43,600, 102,200, and 131,600 CTA units/mg of protein for bovine fibrin plate, human fibrin plate, and human clot lysis assays and ratios of 3.0 and 2.3 for human clot lysis/bovine fibrin plate and human fibrin plate/bovine fibrin plate, respectively. The corresponding ratios at the Fraction 6 stage for this experiment were 3.4 and 2.7: thus there was no further increase in the relative sensitivity of human clot lysis and human fibrin plate assays to tissue activator after gel filtration. Occasionally, human clot lysis/bovine fibrin plate ratios of up to 5 to 6 were seen in column fractions. In those instances, the Fraction 6 ratio was also in this higher range.

Bio-Gel P-150 gel filtration has yielded tissue activator fractions with specific activities as high as 200,000 CTA units/mg. The average specific activity of the most active fractions was generally in the range of 120,000 to 160,000 CTA units/mg by human clot lysis assay. In nine experiments, average recovery of tissue activator activity in column fractions, compared to that of Fraction 6, was 87, 75, and 77% for human clot lysis, human fibrin plate, and bovine fibrin plate assays, respectively.

Polyacrylamide Gel Electrophoresis and Molecular Weight Determinations—A disc polyacrylamide gel electrophoretic pattern of highly purified activator is shown in Fig. 6. The electrophoretic pattern of a pool of Bio-Gel P-150 fractions having specific activity of greater than 100,000 CTA units/mg of protein revealed a single major band and two minor bands with higher migration rates. Tissue activator activity on bovine and human fibrin plates were associated with the major band. A small amount of activity also was detected at the origin of the gel. This could represent another activator species, but more likely is tissue activator associated with large molecular weight material. Further evidence that the major band represented plasminogen activator was obtained when occasionally activator preparations with specific activities of about 200,000 CTA units/mg gave only one band, corresponding to the major band seen in Fig. 6. In these instances, the tissue activator activity of gel slices on bovine fibrin plates corresponded to the position of the single band.

While distinct and separate bands were observed in electro-

FIG. 6. Polyacrylamide gel electrophoresis of activator isolated from preparative column fractions (Fig. 5). The electrophoresis system of Neville (12) was employed and a 50-μl sample containing 11 μg of protein and 670 CTA units of activator was applied. Bio-Gel P-150 column fractions which had specific activities of greater than 100,000 CTA units/mg (human clot lysis) were used.

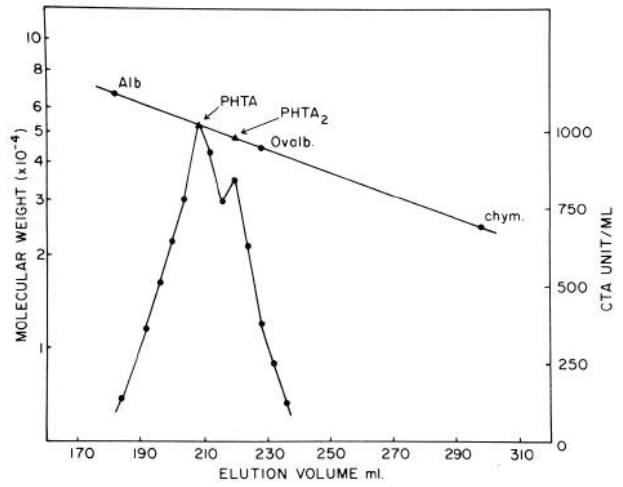

FIG. 7. Estimation of the molecular weight of activator (*PHTA*) by gel filtration on a column (2.5 × 90 cm) of Bio-Gel P-150. Preparation of activator and marker proteins and elution was as described under "Materials and Methods." Marker proteins used were bovine serum albumin (*Alb*), ovalbumin (*Ovalb*), and chymotrypsinogen (*Chym*).

phoretic gels run in the acid system of Neville (12), the alkaline system of Davis (18) did not reveal distinct bands. Diffuse staining of the top one-half of the gel was observed. When gel slices were placed on bovine and human fibrin plates, tissue activator was distributed over the same part of the gel.

Estimation of the molecular weight of tissue activator by gel filtration is shown in Fig. 7. The chromatogram was obtained by gel filtration of Fraction 6 on a preparative Bio-Gel P-150 column, calibrated with marker proteins. The estimated molecular weight of the major activator peak was 52,500, that of the minor peak was 48,000. Gel filtration of an acid/acetate extract of Step 5 activator on columns (1.5 × 56 cm) of Bio-Gel P-150 gave an average molecular weight of 51,500 ± 3,400 for the major component; because of the smaller size of the column, separation was not sufficient to accurately estimate the molecular weight of the smaller species.

Immunological Studies—Quenching of the bovine fibrin plate activity of tissue activator by the γ-globulin fraction of antiserum against activator is illustrated in Fig. 8. Incorporation of γ-globulin in the plate at a final dilution of 1:1000 completely inhibited lysis at all activator concentrations up to 14.5 CTA units/ml. Quenching of activity was observed at all γ-globulin dilutions up to 1:25,000. However, γ-globulin from serum of nonimmunized rabbit at the same dilutions had no effect on activator activity.

Immunoelectrophoretic patterns of Fraction 6 and chromatographed tissue activator are shown in Fig. 9. Three precipitin arcs, representing one cathodic component and two anodic

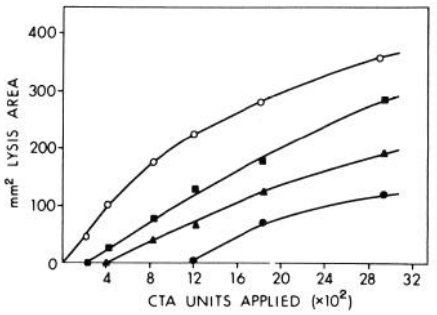

FIG. 8. Inhibition of the biological activity of activator by a γ-globulin fraction of antiserum to activator. Assays were performed on bovine fibrin plates. The *abscissa* shows the concentration of activator solutions used in the assays and the *ordinate* shows the lysis areas produced after 16 h of incubation at 37°. Activator solutions were applied as 20-μl aliquots. γ-Globulin was not present in the control plates, ○——○. Final dilutions of γ-globulin in other plates were 1:5000, ●——●; 1:10,000, ▲——▲; 1:20,000, ■——■.

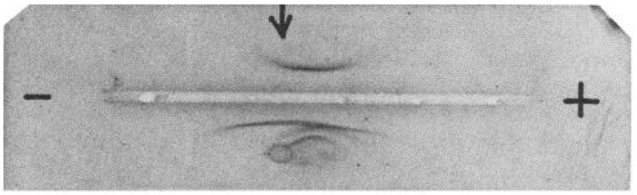

FIG. 9. Immunoelectrophoresis of activator obtained by Bio-Gel P-150 chromatography (*top*) and of Fraction 6 (*bottom*). Each preparation, containing approximately 10,000 CTA units/ml, was applied as a 1-μl spot (*arrow*) to thin gel agarose film, and electrophoresis was conducted at 90 V for 45 min. The trough contained 40 μl of γ-globulin fraction of antiserum to activator. Diffusion was carried out for 24 h, the film washed in 0.9% sodium chloride solution for 3 h and in water for 1 h before staining with 0.125% Coomassie brilliant blue R-250 (18). Destaining was carried out with a 87.5:7.5:5 mixture of water:glacial acetic acid:methanol. The anode is to the *right*.

components, are commonly seen in Fraction 6 patterns. Only one anodic arc was seen in the most purified activator preparation. Similar immunoelectrophoretic studies in fibrin/agarose media established that the anodic band is the active component.

DISCUSSION

Tissue activator of plasminogen is an activity which has been demonstrated in a variety of animal and human tissues (19). Conversion of the zymogen, plasminogen, to the proteolytic form, plasmin, by tissue activator is accomplished by means as yet not elucidated, although it is probably achieved, as with urokinase, through a proteolytic process. The main function of plasmin, a proteolytic enzyme with a high affinity for polymerized fibrin, is the removal of intravascular thrombi. The mechanism for release of tissue activator from tissues has not been well established. Part of the problem has been the difficulty in obtaining sufficient amounts of tissue activator in highly purified form to study its physicochemical properties and to produce specific antisera for localization studies.

The purification procedure described in this report results in the isolation of highly purified activator, representing a 25,000- to 30,000-fold increase in specific activity from that of acetone-dried pig heart. Initial steps of purification, extraction in acid/acetate buffer, ammonium sulfate precipitations at

50% and 35% saturation, and Zn^{2+} precipitation are similar to those reported previously (7) and provide the means for obtaining large amounts of partially purified activator concentrates which are stable for months at −70°, especially in the lyophilized state. The association of activator with other cellular proteins having similar elution characteristics by gel filtration chromatography, and the relative insolubility of activator at neutral pH has hampered progress in the purification of this plasminogen activator for many years. A novel extraction procedure, using buffers containing EDTA, cysteine, and 2,3-mercaptopropanol-1 facilitated removing a large amount of contaminants prior to gel filtration. Zn^{2+}, although useful as a precipitating agent in the purification scheme, reduces the activity of activator, perhaps by binding of activator to other proteins or by blocking an essential sulfhydryl group. The EDTA component of the extracting medium may release activator by chelation of Zn^{2+}, and cysteine and 2,3-mercaptopropanol-1 may further aid in binding Zn^{2+} and by reduction of an essential sulfhydryl group of activator.

In crude preparations of activator an amidase activity is found. This activity is no longer present in Fraction 6 (Fig. 3) and in active activator fractions after gel filtration. The presence of the amidase in crude activator preparations may be responsible for the small amounts of lysis observed on heated bovine fibrin plates. The ratio of lysis activity measured on unheated fibrin plates over that measured on heated plates was approximately 1000:1 in Step 5 preparation; no lysis on heated plates was seen with Fraction 6 or gel-filtered activator concentrates.

It is often difficult to compare the specific activities of tissue activator concentrates prepared in different laboratories. The bovine fibrin plate method was the first to be developed for assay of tissue activator (5), and it has been the method most often used, on the assumption that tissue activator is a direct activator, capable of activating the plasminogen of many animal species. One has to question whether the soluble circulating activator is not a lysokinase, a substance unable to activate bovine plasminogen in the absence of a source of proactivator. In our preliminary investigations of methods for isolation of activator, the discrepancy between human clot lysis, human fibrin plate, and bovine fibrin plate assay values was observed, and consideration had to be given to the possibility that purification of activator was accompanied by the removal of a cofactor of proactivator necessary for activation of plasminogen in bovine, but not for activation of plasminogen in human fibrin plates or in the human clot lysis assay system, conditions which would be analogous to streptokinase activation of various animal plasminogens. This is apparently not the case since recoveries and specific activity increases for the final gel-filtered product as compared to Fraction 1 (Fig. 3) were approximately the same for all three assay methods. A number of other factors could contribute to the differences in activity which were observed when using the three assay methods. The relatively poor solubility at slightly basic pH values and the high affinity of activator to solid fibrin (20) both could lead to poor diffusion of the activator when using fibrin plate assays. Addition of 0.2% albumin to reduce unspecific absorption of activator did not alter assay results.

Of the three methods, the clot lysis method was the most accurate over a wide range of values and identical concentration/lysis curves were obtained for urokinase and tissue activator while log/log plots of activator concentration *versus* lysis zone on fibrin plates, although linear for both urokinase and tissue activator, had different slopes. However, the human clot lysis method has the disadvantage of being unable to measure tissue activator concentrations below 100 CTA units/ml. The data reported here indicate that at any step in the purification process, the measured activity is dependent upon the presence of nonspecific proteases, inhibitors and the physical state of the preparation. Development of a reliable assay may be possible by an immunological method. Immunization of rabbits with the most purified preparations described in this report have elicited antibody production, and a serum γ-globulin fraction from immunized rabbits capable of neutralizing the activator activity has been obtained.

The molecular weight of activator determined by gel filtration of an acid/acetate extract of Step 5 material on small Bio-Gel P-150 columns was 51,500 ± 3,400 for four determinations, which compares favorably with a molecular weight of 52,500 determined for the highest purity activator, chromatographed on the large preparative column. Therefore, treatment of crude activator with chelating and sulfhydryl agents had no effect on the molecular weight of the primary activator component. In both preparations, a second component of the activity peak gave a molecular weight value of 48,000. Although the difference between the molecular weights of the two components is not statistically different, a double peak was observed in eight of nine preparative column runs and in all small column experiments. In some experiments a very small activity peak could also be detected, corresponding to a molecular weight of about 36,000. A molecular weight of 55,000 has been reported for pig ovary tissue activator (8). The two smaller components could be degradation products of the larger component, as has been reported for urokinase (21).

Polyacrylamide gel electrophoresis (acid system) revealed a major and two minor components only. The major component contained measurable activator activity. Because of the insolubility of activator at neutral and slightly basic pH values discrete electrophoretic bands were obtained only under acidic conditions, while optimum detection of activator activity on fibrin plates requires neutral or slightly basic conditions. Application of acidic gel slices probably means that only slices with high tissue activator content will produce lysis areas.

Many questions of the role of tissue activator in the fibrinolytic system can now be answered. A purification method capable of producing tissue activator of high purity will enable investigators to elucidate the physicochemical characteristics of the activator, compare its properties with those of the urinary activator, urokinase, and determine by immunochemical techniques whether circulating blood activator, tissue activator, and urokinase have common identity. With antisera to activator, it is now possible to prepare radioactively labeled antibodies or antibodies conjugated to peroxidase or fluorescent compounds and determine the histological and cytological localization of tissue activator in the animal organism.

REFERENCES

1. Kwaan, H. C., Lo, R., and McFadzean, A. J. S. (1957) *Clin. Sci.* **16,** 241–253
2. Chakrabarti, R., Birks, P. M., and Fearnley, G. R. (1963) *Lancet* **1,** 1288–1290
3. Todd, A. S. (1958) *Nature* **181,** 495–496
4. Astrup, T., and Stage, A. (1952) *Nature* **170,** 929
5. Astrup, T., and Albrechtsen, O. K. (1957) *Scand. J. Clin. Lab. Invest.* **9,** 233

6. Bachmann, F., Fletcher, A. P., and Sherry, S. (1962) *Fed. Proc.* **21,** 64
7. Bachmann, F., Fletcher, A. P., Alkjaersig, N., and Sherry, S. (1964) *Biochemistry* **3,** 1578–1585
8. Kok, P., and Astrup, T. (1969) *Biochemistry* **8,** 79–86
9. Rickli, E. E., and Zaugg, G. (1970) *Thromb. Diath. Haemorrh.* **23,** 64–76
10. Mullertz, A. (1952) *Acta Physiol. Scand.* **26,** 174–182
11. Alkjaersig, N., Fletcher, A. P., and Sherry, S. (1959) *J. Biol. Chem.* **234,** 832–837
12. Neville, D. M., Jr. (1967) *Biochim. Biophys. Acta* **133,** 168–170
13. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412
14. Oliver, D., and Chalkley, R. (1971) *Anal. Biochem.* **44,** 540–542
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
16. Blackwood, C., and Mandl, I. (1961) *Anal. Biochem.* **2,** 370–379
17. Andrews, P. (1965) *Biochem. J.* **96,** 595–606
18. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121,** 404–427
19. Astrup, T. (1966) *Fed. Proc.* **25,** 42–51
20. Thorsen, S., Glas-Greenwalt, P., and Astrup, T. (1972) *Thromb. Diath. Haemorrh.* **28,** 65–74
21. Lesuk, A., Terminello, L., Traver, J. H., and Groff, J. L. (1967) *Thromb. Diath. Haemorrh.* **18,** 293–294