Purification and Properties of a Plasminogen Activator from Pig Heart*

(Received for publication, February 1, 1976, and in revised form, October 28, 1976)

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., Allgaiers, N., and Sherry, S. (1964) Biochemistry 3, 1578-1585). Use of a novel extraction medium containing EDTA, cysteine, and 2,3-dimercaptopropan-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%. Polycrylamide gel electrophoresis revealed a major and two minor components. The molecular weight of the activator determined by gel filtration was 51,500 ± 3,190 for the major activity component and 48,900 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 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

* This work was supported by Grant HL-15817 of the National Heart and Lung Institute, National Institutes of Health, United States Public Health Service.
‡ To whom reprint requests should be addressed.
§ Present address, Office of European Research, Schering Corp., U.S.A., Toepferstrasse 5, 6004 Lucerne, Switzerland.

† 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, and all results are expressed as units/ml. The reproducibility of the lysis time 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 ±8%.

Bovine fibrinogen (Fraction I, 65% clottable) was obtained from Pentex, Kankakee, Ill., and human fibrinogen (90% clottable) from Cutter Laboratories, Berkeley, Calif. For human and bovine fibrin plate assays, the method of Mullertz (10) as modified by Aikjase et al. (11) was used. Tissue activator preparations were diluted in 0.075 M potassium acetate, 0.2 M NaCl, pH 4.2, and applied to fibrin plates as 20-μl aliquots to yield lysis zones between 150 to 300 mm². 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/ml of urokinase/m1 typically produced lysis zones of 250 mm². Reproducibility of bovine fibrin plate and human fibrin plate assays was ±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 determined by hybridization 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.

Discrete 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 the 0.75 mA/tube while stacking and 1.5 mA/tube thereafter. Gels were stained overnight with Coomasie 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 unaided proteins, 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°C. 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 UVcord II detector and recorder unit at 280 nm, and absorbance of each fraction was also measured at 280 nm in a Beckman DU spectrophotometer.

Purification of Tissue Activator

Gel P-150 columns used in the preparative method of activator 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°C acetone in a 6-liter Waring Blender and further homogenized. Samples of dry ice were added to maintain low temperature. Homogenization was performed in Buchner funnels using Whatman No. 541 paper and suction. The filter cake was then added back to the Waring Blender 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°C. 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 at 5°C. 900 ml of ammonium sulfate/liter of extract were added over a 4-h period to give 50% saturation at 2°C. After overnight settling, the settled precipitate was centrifuged and worked into a smooth paste and dispersed into 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 (NH₄)₂SO₄ was added slowly with stirring (200 g/liter, 35% saturation at 2°C). 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: Zn²⁺ Precipitation at Low Ionic Strength—The dialyzed solution was adjusted to pH 4.2 with acetic acid and the sample was then centrifuged. 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 Zn²⁺ 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 solubilized, and then lyophilized. Six hundred to seven hundred grams of acetone-dried pig heart yielded 2.5 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 70:10:10:1 mixture of Triton EDTA/cysteine,2,3-dimercapto-
purification of tissue activator

proponol-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°C 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°C 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°C. 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,O. Four-milliliter fractions were collected by drop counting.

Step 8: Acetone Precipitation of Column Fractions - Active column fractions were pooled and dialysed overnight in the cold against distilled water. The pH was adjusted to 7.0 by addition of 0.1 M NaOH and 10% 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°C to -8°C. The precipitate was recovered by centrifugation at -10°C (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°C.

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°C. 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.
Activity - Fig. 3 outlines the preparative extraction procedure for the human clot lysis activity of tissue activator. This phenomenon involves the fibrin plate method; using the human fibrin plate assay, the activator is separated into the supernatant (Fraction c) and residue (Fraction d) fractions. The supernatant after centrifugation of Fraction a. Little activity was found in the supernatant (Fraction 2), although about 50% of the protein was removed. Resuspension of the sediment in Tris:H$_2$O 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$_2$O 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 8,500 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 bovine fibrin plate and human clot lysis 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 8,500 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).

**Table 1**

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

| Fraction | Total protein | Bovine fibrin plate | Human clot lysis | HCL/BPP* ratio |
|----------|--------------|---------------------|-----------------|---------------|
| Fraction | 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  | 1,190 | 850 | 23,800 | 4,900 | 3,500 | 98,000 | 4.2 |
| d        | 220 | 300 | 28 | 6,000  |        |     |         |     |

* HCL, human clot lysis; BPP, 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 x 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.
Purification of Tissue Activator

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⁺

1 g Step 2 activator
Suspend in 180 ml
Tris:EDTA:cysteine:2,3-mercaptopropanol-
\[ \text{pH 7.4} \]

Fraction 1
homogenize, stir 20 min, centrifuge 30 min at 9000 \( \times g \)

Supernatant
Fraction 2
Residue, suspend in 180 ml Tris:H₂O₂, pH 7.4
Fraction 3
homogenize, stir 20 min, centrifuge 30 min at 9000 \( \times g \)

Supernatant
Fraction 4
Residue, suspend in 20 ml 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2
Fraction 5
homogenize, stir 30 min, centrifuge 30 min at 13,500 \( \times g \)

Supernatant
Fraction 6
Residue, suspend in 10 ml 0.075 M potassium acetate, 0.3 M NaCl, pH 4.2
Fraction 7
homogenize, stir 30 min, centrifuge 30 min at 13,500 \( \times g \)

Supernatant
Fraction 8

Fig. 3 Preparative fractionation scheme employing EDTA/sulfhydryl and acid/acetate extraction.

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.

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 plate 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⁺⁺-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°C. 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 mm², 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 assay values of activator fractions obtained by preparative extraction procedure

| Fraction | Total protein (mg) | Bovine fibrin plate (U/ml) | Human fibrin plate (U/ml) | Human clot lysis (U/ml) | Ratio* |
|----------|-------------------|--------------------------|--------------------------|------------------------|--------|
|          |                   | Total U                   | U/ml                      | Total U                | U/ml   | Total U                |
| 1        | 1,040             | 390                       | 67                       | 76,800                 | 640    | 115,200                | 2.8  | 1.6                   |
| 2        | 615               | 12                        | 5                        | 2,040                  | 25     | 1,560                  | 3.0  |                      |
| 3        | 445               | 495                       | 236                      | 89,100                 | 740    | 133,700                | 2.2  | 1.5                   |
| 4        | 76                | 0                         | 0                        | 0                      | 9      | 1,560                  | 2.2  | 1.5                   |
| 5        | 265               | 4,380                     | 332                      | 87,600                 | 370    | 97,600                 | 0.7  | 1.1                   |
| 6        | 13                | 1,730                     | 2,480                    | 32,100                 | 370    | 59,200                 | 0.7  | 1.1                   |
| 7        | 245               | 1,330                     | 54                       | 13,400                 | 57     | 13,800                 | 0.4  | 1.0                   |
| 8        | 3                 | 840                       | 2,270                    | 7,140                  | 1,370  | 11,700                 | 3.2  | 1.6                   |

* HCL, human clot lysis; BFP, bovine fibrin plate; HFP, human fibrin plate; U, units.
Purification of Tissue Activator

TABLE III
Initial enzyme purification steps

| Step | Product | Human fibrin plate assay | Bovine fibrin plate assay | HFP/BFP |
|------|---------|--------------------------|--------------------------|---------|
| 1    | Acetone powder | 5 | 1,834,400 | 0 | 1,999,100 | 1.0 |
| 2    | pH 4.2 Acetate extract | 24 | 1,632,200 | 84 | 11 | 752,200 | 38 | 2.2 |
| 3    | 50% (NH₄)₂SO₄ ppt. | 43 | 1,247,600 | 76 | 18 | 522,100 | 69 | 2.4 |
| 4    | 35% (NH₄)₂SO₄ ppt. | 65 | 1,008,200 | 81 | 31 | 501,400 | 96 | 2.9 |
| 5    | Zn⁺⁺ ppt., dialyzed, lyophilized | 186 | 462,700 | 46 | 89 | 220,600 | 44 | 2.1 |

% yield, Step 1 to Step 5
% yield, Step 2 to Step 5

a HFP, human fibrin plate; BFP, bovine fibrin plate; U, units.
b Suspended in pH 4.9 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/sulphydryl compounds and acid/acetate by the preparative fractionation scheme (Fraction 6 of Fig. 3) on a column (2.5 x 90 cm) of Bio-Gel P-150. Representing 830 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 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-
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 antiserum 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
bovine fibrin plate method was the first to be developed for
activator while log/log plots of activator concentration versus lysis
curves were obtained for urokinase and tissue activator. Accurate over a wide range of values and identical concentra-
absorption of activator did not alter assay results.

Plate assays. Addition of 0.2% albumin to reduce unspecific
blood absorbance of activator when using fibrin
activity which were observed when using the three assay
methods. The relatively poor solubility at slightly basic pH
final gel-filtered product as compared to Fraction 1 (Fig. 3)
the case since recoveries and specific activity increases for the
purification of activator was accompanied by the removal
of proteins or by blocking an essential sulfhydryl group. The
EDTA component of the extracting medium may release activ-
or by chelation of Zn++, and cysteine and 2.3-mercapto-
panol-1 may further aid in binding Zn++ 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)
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 ani-
mal species. One has to question whether the soluble circulat-
ing activator is not a lysokinase, a substance unable to acti-
bovine plasminogen in the absence of a source of proacti-
vator. 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 plas-
minogen 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 activa-
tion 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 concentra-
tions. Lysis curves were obtained for urokinase and tissue activ-
ator 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 physi-
cal 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 y-globu-
lin 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 com-
ponent. 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 compo-
ents 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 compo-
ent, 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 insol-
ubility 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. Ap-
lication 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 fibrino-
ytic system can now be answered. A purification method capable of producing tissue activator of high purity will enable investigatores to elucidate the physicochemical characteristics of the activator, compare its properties with those of the
urinary activator, urokinase, and determine by immuno-
chemical techniques whether circulating blood activator, tis-
ue activator, and urokinase have common identity. With antisera to activator, it is now possible to prepare radioac-
tively labeled antibodies or antibodies conjugated to peroxi-
dase or fluorescent compounds and determine the histolog-
ical 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
Purification of Tissue Activator

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, 174-182
12. Neville, D. M., Jr. (1967) Biochim. Biophys. Acta 132, 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, 440-442
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, 379-379
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. (1969) Fed. Proc. 25, 42-51
20. Thorsen, S., Glas-Greenwalt, P., and Astrup, T. (1972) Thromb. Diath. Haemorrh. 28, 65-74
21. Lesak, A., Terminello, L., Travci, J. H., and Grof, J. L. (1967) Thromb. Diath. Haemorrh. 18, 293-294
Purification and properties of a plasminogen activator from pig heart.
E R Cole and F W Bachmann

J. Biol. Chem. 1977, 252:3729-3737.

Access the most updated version of this article at http://www.jbc.org/content/252/11/3729

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/11/3729.full.html#ref-list-1