Partial Purification and Properties of the Plasminogen Activator from Pig Heart*

FEDOR BACHMANN, ANTHONY P. FLETCHER, NORMA ALKJAERSIG, AND SOL SHERRY

From the Department of Medicine, Washington University School of Medicine, St. Louis, Mo.

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A method is described for the purification of the plasminogen activator from pig heart. The activator could be extracted from acetone-dried pig heart powder with 0.3 M potassium acetate, *p*H 4.2. Subsequent steps involved ammonium sulfate precipitations at *p*H 4.2 and 50% saturation, and at *p*H 8.0 and 35% saturation, followed by precipitation at low ionic strength in the presence of 0.3 mM Zn^{2+}. Further substantial purification was obtained by gel filtration through Sephadex columns and precipitation with acetone. The method was reproducible and gave overall yields from 15 to 25%. The final preparations exhibited specific activities of 12,000–25,000 CTA (Committee on Thrombolytic Agents, provisional standard) units/mg of protein, representing an approximately 1700-fold purification compared to the crude extract. An $s_{20,w}$ value of 3.36–2.77 S was determined at 4 mg/ml. Immunochemical analysis and starch-gel electrophoresis revealed preparation heterogeneity, K_d, of the activity peak on Sephadex G-100/G-200 was 0.28 compared to 0.26 for human albumin monomer and 0.32 for human cyanmethemoglobin. Solubility and stability were optimal at *p*H 2–4. Studies on the activation of purified plasminogen by tissue activator suggest first-order enzyme kinetics.

The plasma, extracellular fluid, and tissues of man and other vertebrate animals contain an enzyme-precursor plasminogen which, on activation by specific kinases, is converted to the proteolytic-enzyme plasmin. The plasminogen-plasmin system, because of its significance in the *in vivo* turnover of fibrinogen and fibrin, has been the subject of considerable study; the current status of the field has been summarized by several groups (Sherry *et al.*, 1959; Alkjaersig *et al.*, 1959a; Von Kaulla, 1963). Though the mechanism of plasminogen activation is relatively well understood (Alkjaersig *et al.*, 1958a,b; Kline and Fishman, 1961), investigations of kinases involved in the activation, particularly those extractable from human or animal tissue, are less advanced.

In 1947 Astrup and Permin reported that tissue fragments would activate plasminogen and produce zones of lysis when incubated on unheated fibrin plates; attempted extraction of the plasminogen activator from the tissues with water or saline solution was unsuccessful. Other investigators, using a variety of solvent systems (Permin, 1947, 1950; Loomis, 1950; Lewis and Ferguson, 1950) reported similar difficulties in the extraction of plasminogen activator from tissue (for convenience this kinase will be subsequently designated "tissue activator"). Later Astrup and Stage (1952), using 2 M potassium thiocyanate, obtained a superior yield of soluble tissue activator, as did Bierstedt (1955a,b), using a magnesium sulfate–sodium chloride extractant. Numerous reports, employing the 2 M KSCN extraction procedure, indicate that tissue activator is present in most animal tissues, high concentrations having been found in porcine heart muscle and in the uterus, adrenal glands, lymph nodes, and prostate of man (Albrechtsen, 1959).

Despite the ease with which tissue activator may be extracted by 2 M KSCN, little progress has been made in purification, for the solvent is a highly polar one and the activator, in the absence of KSCN, has exhibited relative insolubility in neutral-*p*H-range buffers. However the use of a nonpolar extractant, 5 M urea at neutral *p*H, permitted ammonium sulfate fractionation. The final product, unstable on storage, exhibited an approximately 500-fold increase in specific activity (Bachmann *et al.*, 1962). This report describes an improved purification procedure for tissue activator from pig heart and the properties of the preparation obtained.

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EXPERIMENTAL PROCEDURE

Materials.—Potassium acetate buffers were prepared from glacial acetic acid and the *p*H was adjusted with KOH. Bovine fibrinogen (Pentex Co., Kankakee, Ill.) was 65% clottable by bovine thrombin (100 NIH units/mg, Parke, Davis & Co., Detroit). Human urokinase, 40,000 CTA[1] units/mg protein, was kindly supplied by Dr. M. Mozen, Abbott Laboratories, North Chicago, Ill., and diluted for use in buffers containing 0.5% gelatin. Purified human plasminogen (Cutter Laboratories, Berkeley, Calif.) contained 23 casein units/mg protein. Other chemicals were of reagent grade.

Assay Methods.—The potency of tissue-activator preparations was expressed in CTA urokinase units by comparison with a standard-reference urokinase preparation, using both fibrin-plate and clot-lysis assays.

The fibrin-plate assay (Mullertz, 1952) was modified as described by Alkjaersig *et al.* (1959b). Twenty mg of bovine fibrinogen in 6 ml 0.1 M borate buffer, *p*H 8.0, 2 ml of 6% dextran (Abbott Laboratories), 2 ml of 0.7% $CaCl_2$, and 15 NIH units of thrombin were used for each 10 × 10-cm plate. Four different 0.02-ml sample aliquots, diluted to yield lytic zones above and below 200 mm^2, were applied. Preparation potency was calculated from the extrapolated dilution factor yielding a 200-mm^2 lysis zone (typically produced by a reference urokinase solution containing 2.5 CTA units/ml). A plot of log (sample-dilution factor) against log (lysis area) was linear over the range of 100–600 mm^2. Assays for proteolytic activity were performed on fibrin plates, heated to 80° for 30 minutes to destroy contaminating plasminogen.

Two clot-lysis-assay systems were used; the first, suitable for the assay of low activities, involved determination of the degree of dilution of the preparation that would produce 50% clot lysis in 16 hours at 37°. The clot was formed from 1.0 ml of bovine fibrinogen (10 mg/ml in 0.05 M barbital buffer, *p*H 7.6), 0.2 ml of specimen dilution, and 0.3 ml of thrombin (10 NIH

[1] Provisional standard adopted by the Committee on Thrombolytic Agents of the National Heart Institute.

units/ml). Depending upon the fibrinogen batch, 50% clot lysis was produced by 1.5–3.0 CTA urokinase units/tube. Solutions of higher activity were assayed in a human plasma–clot system comprising 0.1 ml of specimen added to 0.2 ml of pooled outdated bank plasma at 37° and clotted with 0.1 ml of thrombin (20 NIH units/ml). The reciprocal of the lysis time, plotted as 100/lysis time in minutes against activator concentration, yielded a standard curve usable over 6 to 20-minutes lysis times, equivalent to an activity range of 250–600 CTA urokinase units/ml.

Plasminogen and plasmin were assayed by casein hydrolysis, employing the method and unitage described by Alkjaersig *et al.* (1959b).

PROTEIN DETERMINATIONS.—The ultraviolet-absorption method (Layne, 1957) was used, and protein concentration calculated from the ratio at 280 and 260 mμ in acetate buffers at *p*H 4.2, using the equation: protein in mg/ml = $1.45\ A_{280} - 0.74\ A_{260}$. Specific activities were expressed as CTA units/mg of protein. Micro-Kjeldahl nitrogen determinations, and carbohydrate assays were performed as described by Kabat and Mayer (1961). Starch-gel electrophoresis was carried out with a modified vertical method (Boyer and Hiner, 1963) using different buffer systems (Smithies, 1959; Poulik, 1957; Ferguson and Wallace, 1961; Askonas, 1961). In some gels 2-mercaptoethanol was added to the gel buffer in concentrations of 5 mM and 10 mM.

Rabbits were immunized, using for each injection 3–5 mg Zn-precipitated protein (step 4 in procedure) suspended in 2% alginate–0.67% calcium gluconate adjuvant (Colab, Chicago Heights). The antigen mixture was divided between the four food pads. The immunization course comprised five injections administered at 3-week intervals. Crude immuno-γ-globulin was prepared by 33% ammonium sulfate precipitation. Ouchterlony immunodiffusion was performed for 4 days at room temperature in 2% Ionagar in 0.1 M potassium acetate, *p*H 4.2, containing 0.5% sodium azide.

Analytical ultracentrifugation was performed with a Spinco Model E apparatus. Photographic plates were projected and measured with a Nikon Shadowgraph, and calculations were performed with the usual corrections (Schachman, 1959).

ENZYME PURIFICATION

All purification procedures were carried out at 2–4°. Measurements of *p*H and resistance were performed at room temperature. Unless otherwise stated, centrifugation was performed in a Lourdres LRA-1 refrigerated centrifuge at 4° and 7000 × *g* for 15–30 minutes.

Step 1: Preparation of Crude Extract.—Pig heart (10 kg) from freshly killed animals was collected in ice and prepared immediately. Blood coagula, the bulk of connective tissue, and fat were removed mechanically. Acetone-dried powder (1100–1200 g) was prepared from 8 kg of cubed muscle by three successive grindings of aliquots in a 6-liter Waring Blendor with a total of 120 liters of −15° acetone. Filtrations were performed using Buchner funnels (Whatman No. 50 paper) with slight suction. The final filter cake was fragmented through a coarse steel sieve and thinly spread on trays; it dried within 30 minutes at room temperature to a fine, light, red-brown powder. Insufficient acetone treatment resulted in slow drying, a brown-black product, and activity loss. The acetone powder was successively extracted with continuous stirring for at least 6 hours, first with 15 liters and then with 10 liters of 0.3 M potassium acetate, *p*H 4.2. Following an 8-hour settling period, the supernatants were recovered by filtration through Whatman No. 4 paper and centrifugation. The combined supernatants totalled 17–20 liters of a red-brown slightly turbid solution.

Step 2: First Ammonium Sulfate Precipitation.—Finely powdered $(NH_4)_2SO_4$, 300 g/liter extract (50% saturation at 2°) was added with stirring over 4 hours. After overnight settling, the turbid supernatant was siphoned off and spun through a Lourdes continuous-flow centrifuge with CFR-1 rotor (17,000 rpm, flow rate 50 ml/min); the precipitate was combined with the main precipitate recovered by conventional centrifugation. The precipitates were worked into a smooth paste and dispersed into 3 liters of distilled water, and the *p*H was readjusted to 4.2 with 1 M acetic acid. The suspension was stirred for 2 hours and centrifuged. The residual sediment was re-extracted overnight with 2 liters of 0.1 M potassium acetate, *p*H 4.2. Following centrifugation, both eluates were combined.

Step 3: Second Ammonium Sulfate Precipitation.—The eluates were brought to *p*H 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 750 ml of 0.05 M acetic acid, was dialyzed overnight against 25 liters of distilled water.

Step 4: Zn^{2+} Precipitation at Low Ionic Strength.—When the resistance of the dialyzed solution exceeded 1000 ohms, the sample was cleared by centrifugation, the *p*H of the supernatant was adjusted to 6.0 with 1 N NaOH, and a solution of 10 mM zinc acetate was added slowly to a final Zn^{2+} concentration of 0.3 mM. The *p*H was adjusted to 6.5 with 0.1 M sodium barbital, and stirring was continued for 15 minutes. One hour later the precipitate was recovered by centrifugation and dispersed into 220 ml 0.075 M potassium acetate–0.3 M NaCl, *p*H 4.2, with stirring for 3 hours. Insoluble material was removed by centrifugation and the supernatant was saved.

Step 5: Gel Filtration on Sephadex G-100 and Acetone Precipitation.—A large Lucite column (8.8 × 120 cm) of Sephadex G-100 (bed volume, 7400 ml) was previously equilibrated with a minimum of 30 liters of 0.075 M potassium acetate–0.3 M NaCl, *p*H 4.2; the buffer level in the column was 8–10 cm above the Sephadex level. Solid sucrose was added to the supernatant from step 4 to a concentration of 5% and the sample was fed through a Sigma motor pump and applied by a capillary tube about 0.5 cm above the Sephadex level. When all of the sample had entered the Sephadex gel, the buffer inflow was connected to the column with a hydrostatic pressure of 40 cm H_2O. Flow rate was between 100 and 150 ml/hour. Protein elution was monitored with a Vanguard automatic ultraviolet analyzer. The first protein portion appeared after 1.8–2.0 liters, with a peak at 2.3–2.5 liters of effluent. Tissue-activator activity appeared in low concentrations with the main protein peak, but increased significantly when the absorption values of the main protein peak were declining; the maximum activity was eluted at effluent volumes of 2.8–3.3 liters. The fractions were collected in 80- to 100-ml amounts. Activity and absorbancy were determined in all portions, and fractions showing an activity of 200 CTA units/ml or more were dialyzed immediately against distilled water for 4 hours. The activity was labile at this stage (30–50% losses in 24 hours at 4°) and the next step was performed without delay. The pooled dialyzed fractions had a volume of 300–400 ml and an average absorbancy at 280 mμ of 0.2–0.3. With solution resistance between 250 and 350 ohms, *p*H was brought to 6.0 with 1 N NaOH, and finally to 6.5 with

TABLE I
ENZYME PURIFICATION

Step	Fraction	Volume (ml)	Total Protein (g)	Fibrin-Clot Method: Activity (CTA units/ml)	Specific Activity (CTA units/mg)	Total Activity (CTA units)	Yield (%)	Purification Factor	Fibrin-Plate Method: Activity (CTA units/ml)	Total Activity (CTA units)	Absorbancy Ratio (280/260)
(1)	Crude extract	18,500	90	45	9	830,000	100	1	5	92,500	0.7
(2)	First $(NH_4)_2SO_4$ precipitate	3,200	29.5	220	24	705,000	85	2.7	190	608,000	1.2
(3)	Second $(NH_4)_2SO_4$ precipitate dialyzed	800	16.8	800	38	640,000	77	4.2	900	720,000	1.5
(4)	Zn precipitate	200	4.2	3,600	170	720,000	87	19	3200	640,000	1.4
(5)	First Sephadex-gel filtration and acetone precipitation	10	0.1	40,000	4,000	400,000	48	444			1.5
(6)	Second Sephadex-gel filtration and acetone precipitation	1.5	0.01	105,000	15,000	157,000	19	1670			1.5

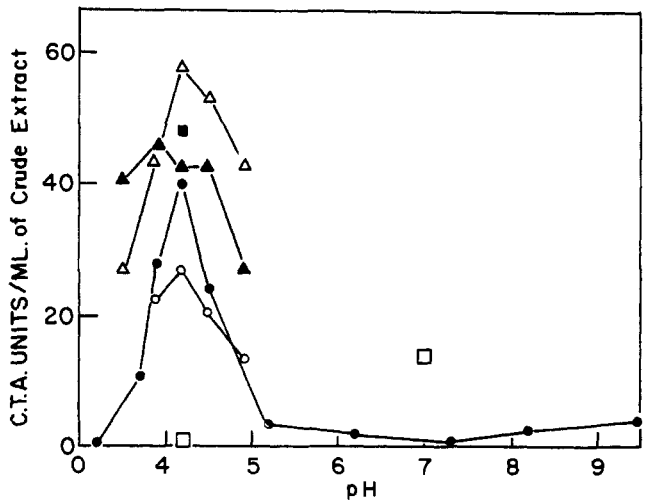

FIG. 1.—Influence of *p*H and salt composition on yield of tissue-activator activity from pig heart. Fresh pig heart muscle (20 g) was homogenized with 100 ml of designated solution and centrifuged after any necessary *p*H adjustments with 1 N NAOH or 1 N HCl. The supernatant activity was determined with the fibrin-plate method. ●—●, 0.15 M NaCl; □, 2 M KSCN; ○—○, 0.1 M potassium acetate; △—△, 0.5 M potassium acetate; ■, 0.15 M KCl; ▲—▲, 0.3 M potassium citrate.

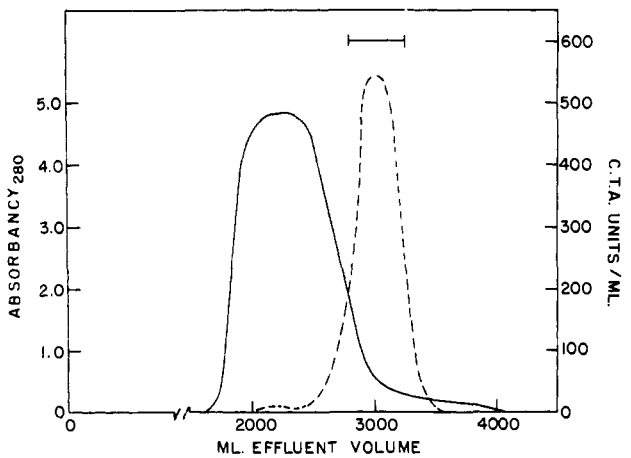

FIG. 2.—Gel filtration of 200 ml step 4 material on an 8.8 × 120-cm column of Sephadex G-100. Elution was performed with 0.075 M potassium acetate–0.3 M NaCl, *p*H 4.2. The activity in the eluate was determined by human clot-lysis assay. Absorbancy at 280 mμ, ——; activity in CTA units/ml, – – –. The graph bar indicates the fractions pooled for step 6.

0.05 M sodium barbital. Over 1.5 hours, with meticulous control of temperature (0 to −8°), −20° acetone was added to a final concentration of 33% (v/v). The precipitate was recovered by centrifugation (7000 × *g* for 30 minutes at −8°) and, after removal of excess acetone with cooled air, was dissolved in 10 ml 0.01 M potassium acetate, *p*H 4.2. After readjustment of the *p*H to 4.2 with 0.5 N acetic acid and 30 minutes' stirring, the solution was clarified by centrifugation at 25,000 × *g* for 10 minutes and stored at 2° or frozen. Freeze-drying resulted in 30–50% loss of activity.

Step 6: Second Gel Filtration on Sephadex G-100/G-200 and Acetone Precipitation.—A further 3- to 4-fold purification could be obtained by gel refiltration on a mixture of 75% Sephadex G-100 and 25% Sephadex G-200. The Sephadex bed volume was 100 times greater than the sample volume. In other respects gel filtration and acetone precipitation were performed as described.

Table I displays data on preparation specific activity and overall yield at each step of a typical purification procedure. The yield at the final stage, 6, calculated by the clot-lysis method, was 19%, and the purification, on a specific-activity basis, was 1670-fold.

RESULTS

Conditions for Extraction from Tissue.—Tissue-activator yield was critically related to tissue-extraction conditions, particularly with respect to *p*H and, to a lesser extent, salt composition of the extractant. Figure 1 shows extract activity as a function of these variables. Activator yield was optimal over the comparatively narrow *p*H range 3.9–4.5, and exceeded that obtained with 2 M KSCN at neutral *p*H (KSCN ineffective as an extractant at *p*H 4.2). Only negligible activity was found in 0.15 M sodium chloride extracts at *p*H 5.2–9.5. A number of buffers including citrate, phosphate, pyrophosphate, barbital, borate, and Tris, in the *p*H range 5–9, were also unsatisfactory. Citrate and acetate buffers were equally effective extractants at *p*H 3.9–4.5, but potassium salts were slightly more effective than sodium salts. Activity yield increased with higher buffer molarity up to an ionic strength of about 0.3–0.5. Addition of 10–50% glycerol, 10–50% ethylene glycol,

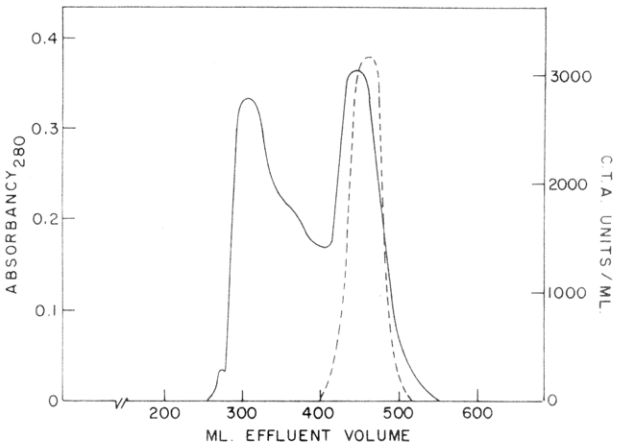

FIG. 3.—Second gel filtration of the active portion from Fig. 2 (sample volume, 10 ml). Bed volume was 3.5 × 100 cm. A mixture of 75% Sephadex G-100 and 25% Sephadex G-200 was used. Elution and activity determinations were performed as indicated in Fig. 2.

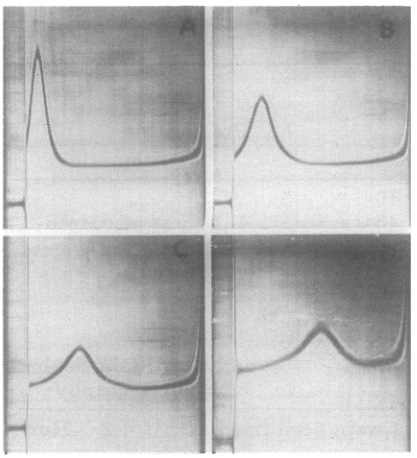

FIG. 4.—Ultracentrifugal patterns of step 6 material (run 5, Table II) after rotor acceleration to 59,780 rpm at 16, and 32 (schlieren angle 60°), 56, and 88 (schlieren angle 50°) minutes.

2% pyridine, 1–5 mM cysteine, 1 mM reduced glutathione, 0.01–0.3 M glycine, or of detergents such as 3 mM hexadecyltrimethylammonium bromide or 0.02–0.5% sodium deoxycholate did not extract significantly higher activities in acetate or citrate buffers. Potassium acetate, *p*H 4.2, 0.3 M, was chosen as the standard extractant because it did not interfere with the following ammonium sulfate precipitation.

Behavior of Material During Purification.—The crude extract showed a low 280/260 absorbency ratio of 0.7, presumably owing to the concomitant extraction of nucleic acids. The numerical ratio increased to 1.5 following ammonium sulfate fractionation (steps 2 and 3, Table I). Later purification steps produced little change in the ratio; that of the final product ranged from 1.5 to 1.7. A 4-fold increase in preparation specific activity was achieved by dissolving the precipitate from step 3 in 0.05 M acetic acid, dialyzing to low ionic strength (resistance 1000 ohms or higher), and precipitating the activator activity at *p*H 6.5, 0.3 mM Zn^{2+} concentration. This latter ion made recovery quantitative. The redissolved precipitate was viscous, and analytical ultracentrifugation revealed the presence of significant contaminations with high-molecular-weight material.

At this stage, the use of various absorption-elution steps (barium sulfate, barium carbonate, calcium phosphate, silica gel, kaolin, charcoal, Florisil, aluminum hydroxide, and bentonite) under a variety of conditions yielded only slight purification or produced serious recovery losses. Similarly, column chromatography with hydroxylapatite, DEAE- and CM-cellulose, DEAE-, CM-, and SE-Sephadex, and Amberlite IRC 50 under various conditions seldom yielded a greater than 4-fold purification and/or was accompanied by poor yield. In contrast, both Sephadex, G-100 and G-200, but not Sephadex G-75, produced good separation of inert proteins from tissue activator, provided that gel bed volume was at least 30 times greater than sample volume. Comparable purification and recovery could be obtained with either grade, though elution peaks were more widely spread with Sephadex G-200. Since flow rates were approximately 3-fold greater with Sephadex G-100, this gel was preferred for large-batch preparation. With both Sephadex grades, material exhibiting absorbency at 280 mμ and dialyzable was eluted at K_d values of 0.9–1.0; the nature of these small molecular products was not investigated. Figure 2 illustrates a typical gel filtration with a 200-ml sample applied to a Sephadex G-100 column of 8.8 × 120 cm. Good separation, resulting in a 12-fold purification, was achieved between the bulk of inert proteins and the activity peak. Separations were dependent on the salt concentration of the elution buffer. Optimal separation, with less than 5% of the total activity eluted with the first peak, occurred at buffer ionic strengths of 0.25–0.5. With buffer ionic strengths between 0.03 and 0.1 at *p*H 4.2, inferior separation was observed. Though column recovery appeared to be quantitative, tissue-activator eluate was unstable and losses occurred at this stage. Moreover rigid temperature control during the acetone precipitation step was crucial for quantitative recovery.

A further 3- to 4-fold purification could be obtained with a second gel filtration. A mixture of 75% Sephadex G-100 and 25% Sephadex G-200 (on a dry-wt basis) produced better separation than did either grade alone. Figure 3 illustrates a typical gel refiltration. Column-bed volume of the Sephadex mixture was 100 times greater than sample volume. The first inert protein peak was eluted at a slightly higher column-volume fraction than the inert peak in the first gel filtration. This peak was small or took the shape of a shoulder when only the most active fractions of the first gel filtration had been pooled. A second peak, comprising all of the activity, was eluted later. Protein absorbancies and activity peak did not coincide; the latter was regularly eluted about 0.03 column-volume fraction later than the absorbancy maximum, suggesting the presence of inert proteins of similar or slightly higher molecular size in this peak. No further increase in specific activity could be obtained by passing the preparation from step 6 through Sephadex for a third time, although ultracentrifugal patterns showed more symmetrical peaks than with material passed only twice through Sephadex. After this step, preparations of a specific activity corresponding to 15,000 CTA units/mg of protein were reproducibly obtained. The highest specific activity ever achieved during our last ten purification procedures with a single gel-filtration fraction corresponded to 25,000 CTA units/mg.

Physicochemical Properties.—Characterization studies were performed with tissue-activator preparations of 4000–20,000 CTA units/mg specific activity.

Sedimentation Behavior.—Analytical ultracentrifugal data on three tissue-activator preparations at various stages of purification are shown in Table II. The purer preparations showed single nonsymmetrical

TABLE II
ULTRACENTRIFUGATION DATA[a]

Run No.	Preparation No.	Preparation Step	NaCl (M)	Specific Activity (CTA units/mg)	Protein Concn (mg/ml)	$s_{20,w}$ (1)	$s_{20,w}$ (2)	Per Cent of $s_{20,w}$ (2)[b]
1	46	(5)	0.15	6,000	13.8	6.88	4.34	72
2	46	(5), + 10 mM mercapto-ethanol	0.15	6,000	13.8	6.47	4.27	76
3	46	(5), lyophilized	0.15	4,000	12.5	6.84	4.27	71
4	46	(6)	0.2	15,000	6.0		3.68	100
5	47	(6)	0.3	14,000	6.0		3.42	100
6	47	(6)	0.3	14,000	3.1		3.36	100
7	37	(6), additional CM-Sephadex chromatography	0.25	18,000	4.0		2.77	100

[a] All runs were performed at *p*H 4.2. Runs 1 through 6 contained 0.05 M potassium acetate; run 7, 0.25 M potassium acetate in addition to indicated NaCl concentration. [b] Percentage total area of the slower-moving peak.

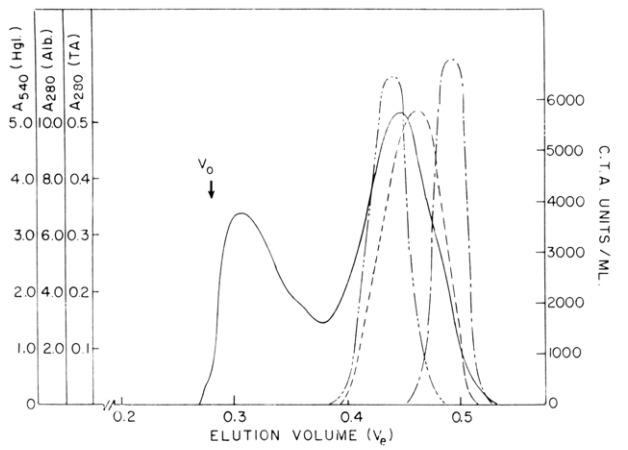

FIG. 5.—Elution profiles of protein and activity peaks from a 2.2 × 70-cm column of 75% Sephadex G-100 and 25% Sephadex G-200. Runs with step 5 preparations and with human-albumin monomer were performed in 0.25 M NaCl–0.25 M potassium acetate, *p*H 4.2; that of human cyanmethemoglobin with 0.15 M NaCl–0.01 M phosphate, *p*H 7.6. The elution volumes V_e are expressed as functions of the bed volume V_t. Void volume V_0, determined with India ink in distilled H_2O, is indicated by arrow. The absorbancy of the tissue-activator preparation and of albumin were read at 280 mμ, that of cyanmethemoglobin at 540 mμ. Tissue activator preparation, ——; human-albumin monomer, — - - —; human cyanmethemoglobin, — · —; activity peak of tissue activator, — — —.

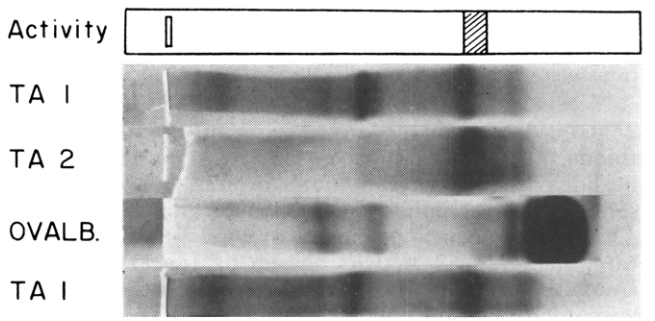

FIG. 6.—Starch-gel electrophoresis of tissue-activator preparations and ovalbumin. A discontinuous buffer system was used (Poulik, 1957) and the gel buffer contained 10 mM 2-mercaptoethanol. The run was performed at 9 v/cm over 6 hours. The crosshatched area indicates the position of tissue-activator activity. TA 1: step 5 preparation, 8000 CTA units/mg. TA 2: same preparation after step 6, 18,000 CTA units/mg. Ovalb: egg albumin, 2 × crystallized, as a reference protein.

inhomogeneous peaks (a representative pattern from run 5 is in Fig. 4) and $s_{20,w}$ values of 2.77–3.68 S were determined at protein concentrations of 3–6 mg/ml.

K_d of Tissue Activator on Sephadex.—Respective K_d values for tissue activator, human-serum-albumin monomer, and human cyanmethemoglobin were determined using a column of 2.2 × 70 cm, filled with a mixture of 75% Sephadex G-100 and 25% Sephadex G-200 (dry wt). Samples were applied in volumes not exceeding 2 ml. The flow rate in all experiments was 5–7 ml/hr. Calculations were performed according to Flodin (1962) and V_0, V_i, V_e, and V_g all expressed as fractions of V_t. Values of $V_i + V_0$ were determined with 50% saturated KCl and were 0.91. This value was corrected to 0.93 since K_d for KCl is 0.98. Using the equation $V_t = V_0 + V_i + V_g$, V_g was calculated as 0.07 and V_i as 0.65. The distribution coefficient K_d of a protein was determined according to the equation $K_d = (V_e - V_0)/V_i$. Collection of the effluent was started when half of the sample had entered the gel and V_e was measured at the maximum peak of the elution profile.

Figure 5 shows the elution profiles of albumin monomer, cyanmethemoglobin, and a partially purified tissue-activator preparation. Tissue-activator activity was eluted between albumin monomer and cyanmethemoglobin, the respective K_d values being 0.26 for human albumin monomer, 0.28 for the activity maximum of tissue activator, and 0.32 for cyanmethemoglobin. Protein and tissue-activator maxima did not coincide, K_d for the protein peak being 0.265.

Electrophoretic Behavior on Starch Gel.—Though several systems were tried (Smithies, 1959; Askonas, 1961; Ferguson and Wallace, 1961, and acetate buffers, *p*H 3.5–4.5), the greatest number of bands were observed when using Poulik's discontinuous buffer system (Poulik, 1957) especially with 10 mM mercaptoethanol (Fig. 6). TA 1 was a step 5 preparation with a specific activity of 8000 CTA units/mg. Six protein bands were clearly distinguished. The activity was all confined to one band with a mobility slightly less than human albumin. TA 2 was obtained from TA 1 after a second gel filtration and exhibited a specific activity of 18,000 CTA units/mg. A broad intense band coincided with the activity band, and there were two faster- and a very faint slower-moving band distinguished. Step 6 material (TA 2) was largely free of contaminating slow-moving bands observed in step 5 (TA 1) material.

Ouchterlony Gel Diffusion.—Figure 7A shows immunodiffusion studies using rabbit antisera and rabbit γ-globulin concentrates obtained after a single immunization course (see Methods) with step 4 material. Step 6 material showed three distinct precipitation lines

TABLE III
INFLUENCE OF *p*H AND SALT CONCENTRATION ON STABILITY OF TISSUE ACTIVATOR[a]

Solution	*p*H	Per Cent Remaining Activity after 24 Hours of Incubation at 4°	20°	37°
1 mM Acetic acid–1 mM NaCl	4.0	100	86	93
1 mM Acetic acid–0.5 M NaCl	3.8	74	62	45
Michaelis buffer	7.2	68	41	40
Borate buffer, 0.1 M	8.0	59	40	41

[a] A step 5 preparation containing 60,000 CTA units and 12 mg of protein/ml in 1 mM acetic acid was diluted 1:30 in the indicated solution and incubated for 24 hours at various temperatures. Remaining activity was assayed after further 1:5 dilution in Michaelis buffer, *p*H 7.2.

with both the antiserum and the concentrated-globulin fraction. However evidence for the formation of neutralizing antibodies was highly questionable; various tissue activator–"antibody" mixtures assayed on fibrin plates and by the plasma-clot-lysis method displayed no loss of activity. One rabbit, after receiving additional immunization, may have developed weak neutralizing antibodies, for 1 mg of rabbit γ-globulin inhibited 40 CTA units of tissue activator by clot-lysis tests, equivalent to 3 μg of a tissue-activator preparation containing 15,000 CTA units/mg. Figure 7B, a diffusion study using antiserum and γ-globulin from this rabbit, demonstrates marked inhomogeneity of step 6 material. At least six bands were discernible against γ-globulin.

Solubility and Stability.—The activity in preparations from steps 1 through 5 was poorly soluble over the *p*H range 5.5–8, particularly at low ionic strength. To determine the amount of tissue activator soluble at physiological *p*H and ionic strength, 0.6 mg of a step 5 preparation with a specific activity of 4200 CTA units/mg was dissolved in 1 ml *p*H 7.38 Michaelis buffer of 0.15 ionic strength and centrifuged for 40 minutes at 25,000 × *g*. Eleven hundred CTA units/ml were recovered in the supernatant, a tissue-activator concentration sufficient to lyse a 1-ml human-plasma clot within 2–3 minutes. Maximum solubility was observed in buffers of resistances of 200 ohms or higher in the *p*H range 2.0–4.5. Tissue-activator preparations obtained after step 5 or 6 were completely soluble in 7% solutions (w/v) in 0.01–1.0 M acetic acid.

Specific activity of step 5 preparations, 7–10 mg/ml in 0.01 M potassium acetate, *p*H 4.2, usually fell 30–50% after 1 month's storage at 2–4°. Storage losses were greater in dilute solutions or at higher salt concentrations. Table III shows activity recovery of a step 5 preparation diluted to 0.4 mg. (2000 CTA units/ml) after incubation for 20 hours at 4°, 20°, and 37° in different solvents. Marked instability was observed at *p*H 8.0, *p*H 7.2, and 0.5 ionic strength at higher temperatures. Not more than 10% losses were observed when preparations were stored at −20° for 1 month.

Other Properties.—Purified tissue-activator preparations exhibited maximum absorbancy between 277 and 278 mμ in 0.1 N HCl, and at 284 and 290 mμ in 0.1 N NaOH. The $E_{280}^{0.1\%}$ of a salt-free step 6 preparation with an activity of 15,000 CTA units/mg dry wt, dissolved in 0.01 M potassium acetate, *p*H 4.2, was 1.0 ± 0.1. Micro-Kjeldahl analysis revealed 16% N/mg of protein. Assay for carbohydrate by the orcinol method, using 0.1 mg of mannose and 0.1 mg of galactose/ml as a standard, showed 1.1% carbohydrate.

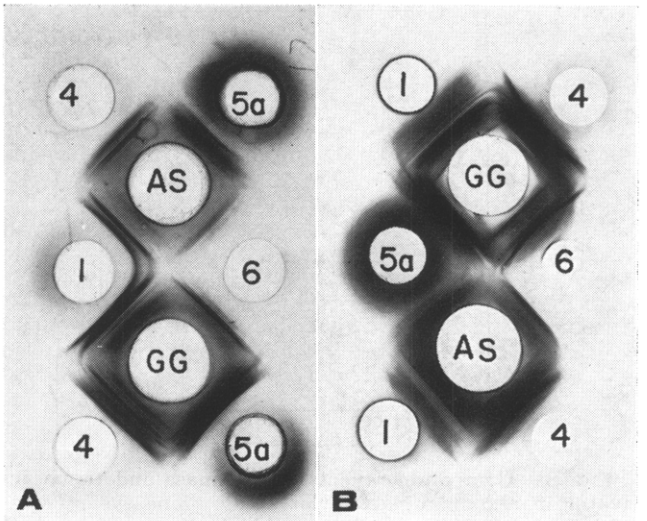

FIG. 7.—Immunodiffusion patterns. Center wells: antiserum (AS) and γ-globulin concentrate (GG) obtained in plate A after a single immunization course and in plate B after further immunization (see text). Antigen wells: crude extract (1), step 4 material (4), first inactive protein peak, obtained during first Sephadex gel filtration (5a), and step 6 material, 18,000 CTA units/mg (6).

Thromboplastic Activity.—Since tissue may contain thromboplastic substances (Biggs and Macfarland, 1962), tissue-activator preparations were tested for their ability to shorten the recalcification time of plasma containing 10^{-2} M ϵ-aminocaproic acid to inhibit plasminogen activation. Tissue activator (100–700 CTA units/ml in the test system) produced no effect on the recalcification time, but higher concentrations tended to increase it. A diluted commercial tissue-thromboplastin (Simplastin, Warner-Chilcott, Morris Plains, N. J.) preparation shortened the plasma-recalcification time from 210 seconds to 190 and 155 seconds at final dilutions of 1/12,000 and 1/40,000, respectively.

Presence of Proteolytic Properties Probably Unrelated to Tissue Activator.—When a urokinase solution containing 5000 CTA units/ml was applied to a heated fibrin plate, no digestion of the denatured plasminogen-free fibrin occurred during an incubation period of 16 hours at 37°. However, all tissue-activator fractions obtained during the purification procedure produced some lysis of the heated fibrin. The quotient of lysis activity measured on unheated plates over that measured on heated plates was approximately 100:1 in the crude extract and during the first purification steps; this increased during the purification to 10^4–10^5:1 (step 5 tissue-activator preparations of 5000 CTA units/ml gave 50 ± 20 mm^2 zones of lysis on heated plates).

Activator Activity.—Tissue activator and urokinase added to human-citrate plasma clotted with thrombin exerted a similar thrombolytic effect on the human-fibrin clot. Figure 8 illustrates the dependence of the reciprocal of lysis time with respect to activator concentration of urokinase and of a step 6 preparation. The activators diluted in phosphate-gelatin buffer yielded virtually identical concentration/lysis curves in this test system. In contrast to this action in human plasma, the activators, when assayed on bovine-fibrin plates, gave dissimilar results. In both instances log (lysis zone) areas were linear with log (activator concentrations), but the line slopes differed; that of urokinase rose more steeply with log activator concentration than did that of tissue activator. These line-slope differences were, at least in part, attributable to the poor solubility, which hindered diffusion, and poor

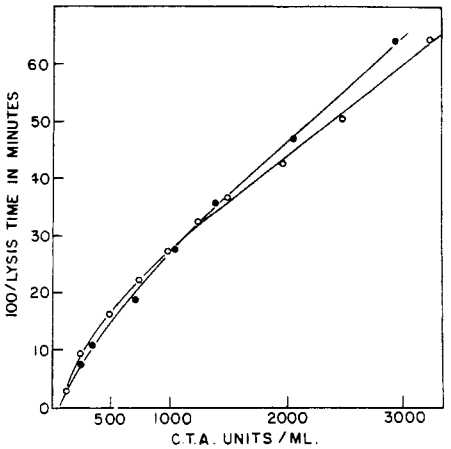

FIG. 8.—Comparable effects of urokinase and tissue activator in a human-plasma-clot assay. The system contained 0.2 ml of pooled human blank plasma, 0.1 ml of activator in the indicated concentration and 0.1 ml of thrombin (20 NIH units/ml) in 0.1 M borate, *p*H 8.0. Urokinase, ○—○; tissue activator (step 6 material), ●—●.

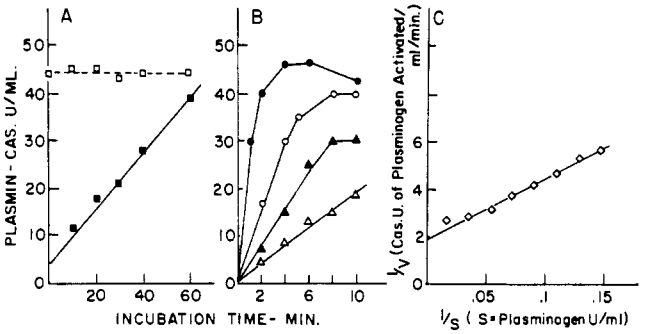

FIG. 9.—Activation of purified plasminogen by tissue activator. (A) Plasminogen, 45 casein units/ml; tissue-activator concentration, 600 CTA units/ml; plasmin, ■; plasmin + plasminogen, □. (B) Plasminogen-concentration constant (46 casein units/ml); tissue-activator concentration varied. 6000 CTA units/ml, ●; 3000 CTA units/ml, ○; 1500 CTA units/ml, ▲; 750 CTA units/ml, △. (C) Tissue-activator-concentration constant (150 CTA units/ml); plasminogen concentration varied within the range 6.75–54 casein units/ml. The incubation period was 15 minutes. In each experiment the activation reaction was stopped prior to assay by the addition of ε-aminocaproic acid to a final concentration of 0.02 M in the assay tube.

stability of tissue activator at neutral *p*H and 37°. Moreover, the fibrin-plate and clot-lysis methods yielded dissimilar results in the assay of crude tissue extracts but not with more purified material (Table I); this may be related to the presence of inhibitors in the crude extracts.

Figure 9 displays data relative to the activation of purified plasminogen by tissue activator (step 5 material). In section A, plasminogen activation by a fixed concentration of tissue activator at 37° increased linearly with time until 90% activation had occurred. The open squares in the diagram represent assays for the total plasminogen and plasmin combined; this parameter was unchanged throughout the experimental period. Section B displays activation rates as a function of time and activator concentration. Initial reaction velocity was proportional to tissue-activator concentration over the range of concentrations tested (750–6,000 CTA units/ml). Section C shows the degree of plasminogen activation as a function of varying substrate concentration over an 8-fold range (6.75–54 casein units/ml) with activator concentration and time kept constant. The $1/v$ was linear with $1/S$. These findings are consistent with the hypothesis that plasminogen activation by tissue activator occurs enzymatically, the reaction following first-order enzyme kinetics.

DISCUSSION

Hitherto, despite current investigative interest in both the biochemical and physiological functions of the plasminogen-plasmin system (Fletcher *et al.*, 1962a,b; Albrechtsen, 1959; Von Kaulla, 1963), study of tissue-plasminogen activator has been hindered by the crude nature of the available preparations. Indeed, because of the problem posed by the virtual insolubility of tissue activator in the usual extractants at neutral *p*H and the difficulties of the tissue extraction itself, reported studies refer either to properties of the material in tissue suspension (Astrup and Permin, 1947; Permin, 1947, 1950; Loomis, 1950; Lewis and Ferguson, 1950) or after its extraction into a 2 M potassium thiocyanate solution (Astrup, 1952; Astrup and Sterndorff, 1956; Albrechtsen, 1957, 1958, 1959; Albrechtsen *et al.*, 1958). The present preparations, though still inhomogeneous by ultracentrifugal, electrophoretic, and immunochemical criteria, represent an approximately 1700-fold purification in terms of the original tissue extract. Though our findings refer to the purification of tissue activator from pig heart, chosen because of its high activator content and its ready commercial availability, we have found that the described purification procedure is applicable to human tissues (lung and heart). Since it has not been possible to obtain sufficient human tissue to prepare activator preparations of comparable purity to those of porcine origin, comparison between these is not yet feasible. Previous investigations have employed thiocyanate extracts assayed on fibrin plates for tissue analysis; yet the data in Table I suggest that the fibrin-plate assay may yield erroneously low values with crude tissue extracts. Preliminary studies with potassium acetate extracts of human tissues support this view and suggest that the reported activator content of human organs (Albrechtsen, 1957) may require upward revision. Tissue activator, as judged by the ultracentrifugal findings, which indicate an $s_{20,w}$ value lying between 3.36 and 2.77 S at protein concentrations of 4 mg/ml and by the results of Sephadex-gel studies, is a comparatively small molecule. These latter studies revealed that tissue activator was eluted from Sephadex G-100 gel in proximity to albumin monomer and between it and cyanmethemoglobin. The significantly higher K_d value obtained for cyanmethemoglobin as compared to albumin was somewhat unexpected. Polson (1961), using 7% granulated agar columns, had attributed this finding to the different molecular-diffusion constants of the two substances; while Andrews (1962), using 9% agar columns, had estimated the molecular weight of bovine hemoglobin as 32,000 and had suggested that hemoglobin had dissociated into half-molecules under these conditions.

Heated-fibrin-plate assays, made after various purification steps, suggest that the action of the cruder preparations on heated fibrin is owing to the presence of a contaminant (possibly cathepsin [s]) and that the purified preparation does not directly attack fibrin. The differential action of the preparations on heated and unheated fibrin plates, the clot-lysis data (Fig. 8), and the kinetic data with purified plasminogen (Fig. 9) demonstrate that the properties of the final preparation

may be explained wholly on the basis of its action as a plasminogen activator.

Though immunization of rabbits with step 4 material elicited antibody production against some preparation components, it is likely that porcine-tissue activator itself is only weakly antigenic in the rabbit. Even after a prolonged course of immunization, antibodies capable of blocking the biochemical actions of the tissue activator were not demonstrated with certainty.

The solubility properties of the present preparations are, at first sight, unusual for an activator which *in vivo* must be inferred to be active at *p*H values in the physiological range. The activator preparation exhibits maximal solubility at approximately *p*H 4 (exceeding 60 mg/ml) and solubility substantially decreases to below 1 mg/ml between *p*H 6.0 and 8.0. Though this relationship of solubility to *p*H could be a preparation artifact arising as a consequence of the purification procedure, the biochemical activity of the preparations (20,000 CTA units/mg of protein), and a demonstrated solubility of 1100 CTA units/ml in Michaelis buffer at *p*H 7.38 indicate that tissue activator exhibits sufficient solubility in physiological media to perform the functions attributed to its activity.

Since it has been suggested that tissue activator, after release from tissue, may circulate in plasma and be excreted in the urine as urokinase (Smyrniotis *et al.*, 1959; Von Kaulla and Riggenbach, 1960; Guest and Celander, 1961), it was of interest to compare the properties of tissue-activator preparations with those of partially purified urokinase. Both possess specific activities of comparable magnitude (25,000 CTA units/mg for tissue activator to 60,000 CTA units/mg protein for urokinase[2]), their sedimentation constants are roughly similar (3.36–2.77 S for different preparations of tissue activator at 4 mg/ml versus approximately 3 S for urokinase examined under similar circumstances[2]), both exert similar thrombolytic action in human plasma (Fig. 8), both are apparently devoid of action on fibrin itself, and limited studies suggest that the kinetics of plasminogen activation by each may be essentially similar. However these data offer only suggestive evidence of similarity and not identity. Studies using synthetic substrates, tosylarginine methyl ester, acetylysine methyl ester (Sherry *et al.*, 1964), and benzoylarginine methyl ester (to be reported separately), suggest that the ratio of activities of tissue activator and urokinase on these substates may be different.

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