The Isolation and Characterization of Plasminogen Activators (Urokinase) from Human Urine*

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ABSTRACT: A method is described for the isolation of two main types of plasminogen activator (urokinase) from human urine. Purification was effected by employing successively the steps of (1) foaming and ammonium sulfate precipitation, (2) adsorption on and elution from IRC-50 resin, (3) gel filtration on Sephadex G-100, (4) refiltration on Sephadex G-100, (5) chromatography on hydroxylapatite, and (6) separate refiltration of each type on Sephadex G-100. The two highly purified main types (S1 and S2) were analyzed by physical, chemical, and immunochemical methods. Type S1 urokinase showed a specific activator activity of 218,000 CTA units/mg of protein and 170,000 CTA units/mg by synthetic substrate assay, and a molecular weight of 31,500 by both the sedimentation–diffusion and sedimentation–equilibrium methods. It was homogeneous by all criteria applied. Type S2 urokinase showed a specific activity of 93,500 CTA units/mg and a molecular weight of 54,700 by the sedimentation–equilibrium method.

Unlike type S1, type S2 urokinase showed a sedimentation–equilibrium plot deviating from linearity, although type S2 was homogeneous by all other criteria. During the course of purification of the two main types, other forms of urokinase were observed, differing from the main types in molecular size (step 3, C2) and in electrical charge (step 5, T). It is conjectured that some of the minor types might be genetic variants, while others might be due to degradation by uropepsin.

The presence in urine of an activator substance capable of effecting the transformation of plasminogen to the proteolytic enzyme plasmin was first described independently by Williams (1951) and in the following year by Astrup and Sterndorff (1952) and Sobel *et al.* (1952). The latter group assigned the name urokinase to this activator.

During the several years following its discovery, methods for limited purification of urokinase were reported (Von Kaulla, 1954; Celander *et al.*, 1955; Ploug and Kjeldgaard, 1957; Celander and Guest, 1960; Sgouris *et al.*, 1960, 1962). More recently, in a brief communication, Lesuk *et al.* (1965) described the preparation of crystalline urokinase. Crystallization was induced in a solution of "purified" urokinase of undefined purification history. The crystalline material was reported to be homogeneous by polyacrylamide gel electrophoresis and ultracentrifugal analyses, but to exhibit reversible association–dissociation reactions. Specific activity determination indicated a level of 104,000 CTA[1] units/mg of protein (the activity presumably being measured by a fibrinolytic assay).

Several years ago, studies were initiated in our laboratories concerned with the isolation and characterization of highly purified urokinase. As an outcome of these investigations, a procedure was developed which resulted in two highly purified urokinase components, one of which exhibits a specific activity of approximately 200,000 CTA units/mg of protein. This communication describes the purification as well as the physical, chemical, and immunological properties of the isolated components.

Experimental Procedures

Materials. Phosphate buffers were prepared from analytical grade reagents using a calculated ratio of $NaH_2PO_4 \cdot H_2O$ and Na_2HPO_4 to obtain the desired molarity and pH. The pH 6.5 buffer referred to in all purification procedures following step 1 is 0.1 M phosphate, pH 6.5, containing 0.1 M NaCl and 0.1% EDTA (disodium dihydrogen Versenate, Dow Chemical). Amberlite IRC-50 resin (Rohm and Haas Co., Philadelphia, Pa) was ground in a Fitz mill, and sieved to 50–100 mesh. New resin was prepared for use by the procedure of Hirs *et al.* (1953), and equilibrated with pH 6.5 buffer prior to use.

Sephadex G-100 for gel filtration (Pharmacia Fine Chemicals, Inc.) was employed with standard techniques. Hydroxylapatite was prepared by the procedure of Tiselius *et al.* (1956), and used fresh.

Assay Methods. Urokinase activator activity was determined by a fibrin tube method developed in our laboratories. Activity is expressed in terms of CTA units (Sherry *et al.*, 1964). In this procedure 0.2 ml of 10 different dilutions (made with 0.1% human serum albumin, fraction V, Pentex, Inc.) of urokinase to be assayed is apportioned into separate tubes. This is

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[1] Standard urokinase unit adopted by the Committee on Thrombolytic Agents, National Heart Institute.

followed in order by 0.3 ml (17 NIH units/ml) of bovine thrombin (Parke Davis, Detroit, Mich.), and 1 ml (16 mg/ml) of fibrinogen (bovine fraction I, Armour Pharmaceutical Co.) dissolved in 0.05 M sodium barbital buffer, pH 7.6. The tubes are then incubated at 37° for 16 hr. If the proper dilutions of urokinase have been used, a graded series of clots will remain, ranging from unchanged to complete lysis. The level of urokinase resulting in 50% clot lysis is estimated, and the activity of the original sample determined by comparison to a standard of assigned potency,[2] which is run simultaneously. Depending on the fibrinogen batch used, 50% clot lysis was produced by 0.25–0.8 CTA urokinase units/tube. Statistical analyses of a series of assays performed on assay standards indicated an estimated error of ±24% from the mean for a single assay at the 95% probability level. Final preparations were assayed at least three separate times, thus reducing the estimated error to about ±16%. The activity of urokinase in catalyzing the hydrolysis of acetyl-L-lysine methyl ester (ALMe)[3] was measured by the method of Sherry *et al.* (1964).

Protein Determinations. For most purification work the protein concentration of solutions was estimated by ultraviolet absorbancy determination at 280 mμ.

Total Nitrogen, Extinction Coefficient, and Amino Acid Analyses. These three analyses were done on the same solution in the case of each of the two most highly purified fractions. A portion of lyophilized protein of approximately 20 mg was taken up in 1 ml of 5% NaCl. The solution was transferred to an 8/32 cellophane casing and dialyzed *vs.* 5% NaCl for 2 days. The bag was then transferred to a large volume of 0.9% NaCl and dialyzed overnight. The almost clear solution was removed from the bag and centrifuged for 30 min at 4° in the Servall RC-2 centrifuge using the SS-34 head at 15,000 rpm. The supernatant was carefully separated from the small amount of insoluble material. Aliquots containing about 2 mg of protein were assayed for total nitrogen using the micro-Kjeldahl procedure. Other aliquots were diluted with pH 6.5 buffer for measurement of 280-mμ absorbancy. The extinction coefficient, $E^{1\%}_{1cm}$, was calculated from these measurements, the protein concentration being estimated as total nitrogen times 6.25.

Aliquots of the same solution, containing 2–3 mg of protein, were hydrolyzed at 110° for 24 and 72 hr according to Hirs *et al.* (1954). The amino acid composition was determined with a Spinco Model 120 amino acid analyzer according to Spackman *et al.* (1958) as modified by Spackman (1964). Tryptophan was determined on samples of intact protein by the spectrophotometric method of Goodwin and Morton (1946).

Ultracentrifugal and Electrophoretic Analyses. Sedimentation experiments were made with the Spinco Model E ultracentrifuge at or near room temperature. Velocity runs were at 59,780 rpm and equilibrium runs at 10,589 rpm. Diffusion coefficients were measured in the Spinco Model H electrophoresis–diffusion apparatus using interferometric optics. Calculations of molecular weights were made by the method of Schachman (1957). From boundary spreading measurements the heterogeneity constant, ρ, was calculated by the method described by Williams *et al.* (1952). Moving-boundary electrophoresis was carried out in the Spinco Model H apparatus in acetate buffer, pH 4.8, $\mu = 0.1$, with a 1.0% protein solution.

Immunochemical Analysis. Rabbits were immunized, using for each injection episode 1–2 mg of protein suspended in 1 ml of complete Freund's adjuvant. The first injection in each series was divided among the four footpads. The second injection was given three weeks later and was applied to the back muscle area. Test samples of sera were withdrawn beginning about 1 week following the second injection of antigen. Booster injections of 1–2 mg of protein were given as needed. Immuno-γ-globulins were prepared by triple ammonium sulfate precipitation (Campbell *et al.*, 1963). In the application of this technique the volume of the solution of purified γ-globulin is adjusted to be one-half that of the original antiserum. Immunodiffusion was performed for 2 days at room temperature using 0.75% Ionagar No. 2 (Consolidated Laboratories, Chicago Heights, Ill.) in pH 7.4 saline containing 0.01% Merthiolate.

To test the quenching activity of the immuno-γ-globulins toward urokinase, serial amounts of the γ-globulins were added to 2000 units of urokinase in 0.1 ml of pH 7.4 saline. The solutions were allowed to stand for 1 hr at room temperature and then were refrigerated until submitted for assay. The residual activity of the solutions was tested in the standard fibrinolytic procedure described above.

Purification of Urokinase

Unless otherwise stated, all purification procedures were carried out at 4°.

Step 1. ISOLATION OF CRUDE UROKINASE. Foam concentrates of urokinase were prepared from human urine essentially by the method of Celander *et al.* (1955). The activity was precipitated by adding $(NH_4)_2SO_4$ to 65% saturation (2°). The $(NH_4)_2SO_4$ precipitate was recovered by centrifugation or filtration, dissolved in 3% NaCl in a volume $^1/_{200}$ of the original urine volume, and dialyzed overnight *vs.* 12 volumes (two changes) of 0.1 M phosphate buffer, pH 6.5, containing 0.1% EDTA. The bag contents were clarified by centrifugation (30,000*g* for 30 min). This solution, designated crude urokinase, had a specific activity of about 250 units/A_{280}.

Step 2. PURIFICATION ON IRC-50 RESIN. Crude urokinase was further purified by adsorption on and elution from Amberlite IRC-50 resin columns by a modified Ploug and Kjeldgaard (1957) procedure. Amberlite IRC-50 resin, previously equilibrated *vs.* pH 6.5 buffer,

[2] Activity standards were lyophilized preparations of purified urokinase whose potency was assigned by the Committee on Thrombolytic Agents, (Abbott Urokinase Assay Standard Lot No. 2021–209.)

[3] Abbreviations used: ALMe, acetyl-L-lysine methyl ester.

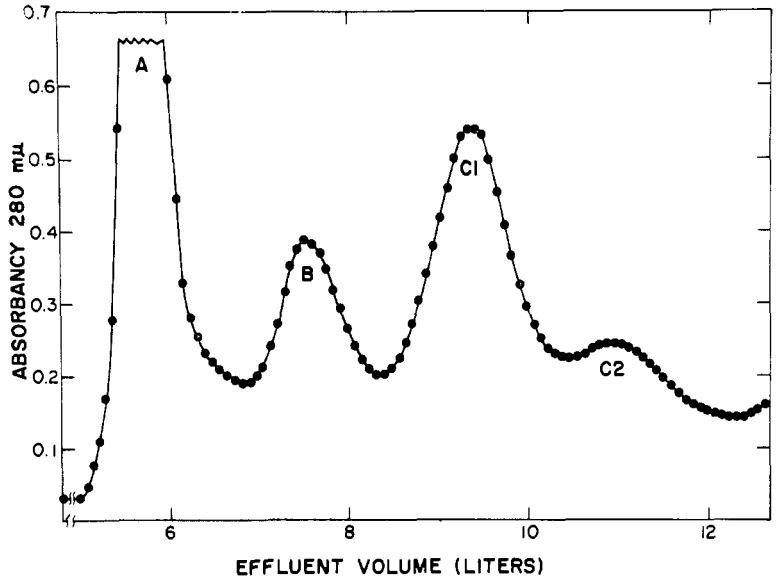

FIGURE 1: Gel filtration of step 2 urokinase. Column size: 15.2 × 110 cm. Equilibrated to pH 6.5 buffer (0.1 M NaCl, 0.1 M phosphate). Sample (5 g, step 2 fraction) applied in volume of 73 ml; flow rate, 200 ml/hr. Fractions cut at low points between peaks except on left side of C-1, where 280 ml was rejected to reduce contamination by peak B. An additional peak, designated D, emerges beyond the right-hand limit of this figure.

was poured as a slurry into a 6-in. diameter column to a packed height of 100 cm. Crude urokinase (100–125 × 10^6 CTA units) was introduced into the column, which was then washed with distilled water until the optical absorbancy of the effluent at 280 mμ was $\leq$0.06. The adsorbed urokinase was eluted with 3% NaCl solution containing 0.1% EDTA. The optical absorbancy of the fractions at 280 mμ was determined, and from these data two pools of activity were made. The active fractions emerging first were combined as pool A. This contained 50–60% of the starting crude urokinase activity at a specific activity of 9,000–10,000 units/A_{280}. The second pool (fraction B) was composed of the more tightly bound activity, and comprised 10–12% of the starting activity at 3500–4000 units/A_{280}. The two-pooled active fractions were either directly lyophilized or first concentrated by ultrafiltration and then lyophilized.

Step 3. GEL FILTRATION ON SEPHADEX G-100 COLUMNS. Figure 1 shows a typical large-scale gel filtration which was done with step 2 fraction A material in a standard 4-ft length of 6-in. Pyrex pipe, using a 325 mesh stainless steel screen supported by a perforated Teflon plate at the bottom. The clarified sample (approximately 5 g of solids with a total activity of 50 × 10^6 CTA units) in a volume of 73 ml, having been equilibrated by dialysis *vs.* the pH 6.5 buffer, was layered carefully on top of the column, the surface of which was stabilized by means of a Whatman No. 1 paper disk held in place by a perforated Lucite plate. Filtration was by gravity alone with the buffer head not >20 in. above the top of the Sephadex bed. The flow rate was about 200 ml/hr. In addition to the four peaks shown in Figure 1, continued elution resulted in the emergence of a fifth peak, designated D, the beginning of which is shown on the right-hand edge of Figure 1. Bioactivity was found only in the region under the peaks labeled C-1 and C-2. The total yield of activity has been in the range of 80–85%. Pools were made of the two active fractions, rejecting 10–12 tubes from the start of peak C-1 to reduce contamination from the inert B peak. The pools were concentrated by ultrafiltration through cellophane casing. Ordinarily, C-1 type material constituted at least 70% of the total biological activity contained in the parent step 2A fraction, and exhibited a specific activity in the range of 30,000–35,000 units/A_{280}. In the remainder of this paper consideration will be given only to the C-1 fraction; the C-2 material is being held for future study.

Step 4. REFRACTIONATION ON SEPHADEX G-100. The concentrated C-1 fractions from six step 3 columns were pooled and further concentrated by ultrafiltration to 75 ml. The solution was clarified by centrifugation and introduced into a standard 6-in. column as before. A large main peak of 280-mμ absorbancy was obtained emerging again in the range of 9–10 l. of effluent volume. Fringe portions of material of low potency, representing about 15% of the applied sample, were eliminated from the peak B and peak C-2 regions. The material under the main peak was then pooled and concentrated as before. In this step the potency of the product rose to 45,000–60,000 CTA units/A_{280}.

Step 5. SEPARATION ON HYDROXYLAPATITE. The step 4 product was further purified and separated into two active forms by chromatography on hydroxylapatite. The hydroxylapatite was poured into a glass column

with a conical bottom plugged with a small wad of glass wool held in place with a layer of glass beads. The column was equilibrated to 0.05 M pH 6.8 phosphate buffer by passing such a solution through the column until the conductivities of effluent and influent were identical. Chromatography was done at room temperature. Figure 2 illustrates such a column. Before chromatography, the sample was equilibrated in solution by dialysis *vs.* the 0.05 M buffer. As seen in the figure, an unretarded peak of 280-mμ absorbancy emerged without change of buffer concentration. In trial experiments, no further 280-mμ absorbing material emerged even with five additional column volumes of 0.05 M buffer. However, application of a gradient at the point shown resulted in the e ution of two additional peaks. Bioassay of the fractions revealed that peak R was inert, while both peaks S1 and S2 were active. Total recoveries of 280-mμ absorbancy and of bioactivity were about 85%. The ratio of total bioactivity under peak S1 to that under peak S2 was about 3:1. In order to avoid cross-contamination, representative pools of fractions S1 and S2 were cut near the center of the peaks as shown in Figure 2. A summary of the results is shown in Table I. The pooled fractions were

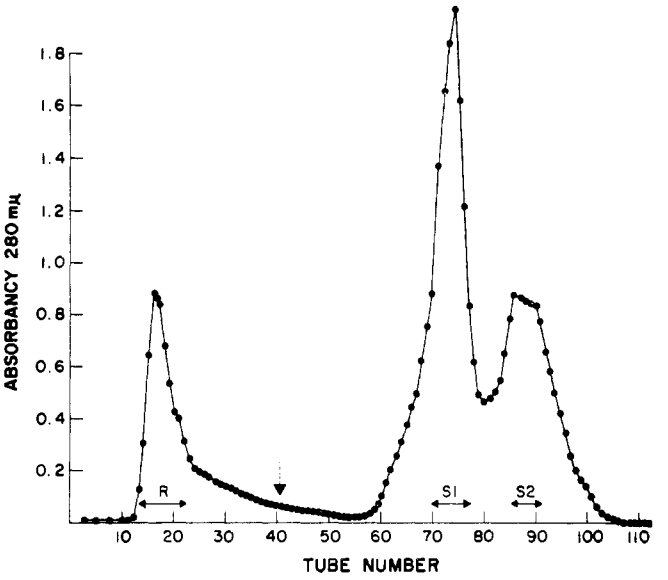

FIGURE 2: Hydroxylapatite chromatography of step 4 urokinase. Column size: 5.8 × 10 cm. Equilibrated *vs.* 0.05 M sodium phosphate buffer, pH 6.8. Volume fractions were 20 ml/tube. Gradient applied at tube 41 by passing 0.25 M buffer into mixing chamber containing 700 ml of 0.05 M buffer. The main fractions are indicated on the graph (see Table I for complete data).

TABLE I: Summary of Hydroxylapatite Purification of Step 4 Material.

Fraction	Tubes	Total A_{280}	Bioact. (CTA Units × 10^6)	Sp Act. (CTA Units/A_{280})
Starting material	...	722	53.1	73,500[a]
R	13–22	91	<0.3	...
(Pre-S1)	62–69	70	4.5	64,300
S1	70–77	222	26.3	119,000
(S1–S2)	78–84	76	3.3	44,000
S2	85–91	119	6.9	57,600
(Post S2)	92–97	55	2.2	40,000
Total recoveries		633 (88%)	43.2 (81%)	

[a] Although the specific activity of step 4 material typically is in the range 45,000–60,000 CTA units/A_{280}, the starting material in this particular experiment assayed higher as indicated.

dialyzed overnight *vs.* large volumes of wa er and lyophilized. In analytical rechromatography on hydroxylapatite columns, the S1 and S2 fractions maintained their original identities, and little or no cross-contamination was evident.

Step 6. REFILTRATION ON SEPHADEX G-100. Fractions S1 and S2 were each subjected to gel filtration on a small Sephadex G-100 column in the same buffer solution used for step 3. The columns had conical bottoms plugged with glass wool held in place by a layer of glass beads. The sample was taken up in pH 6.5 buffer and carefully layered on the column by means of an eye dropper. Filtration was done by gravity, keeping the buffer level a few inches above the top of the column. The results are shown in Figures 3 and 4. In both cases a major symmetrical 280-mμ absorbancy peak resulted, and in each case bioactivity was associated only with the main peak. Total recoveries of bioactivity were substantially quantitative. The fractions were pooled as indicated in the legends, and each was dialyzed overnight against water and lyophilized.

Results

Evidence for Heterogeneity at IRC-50 Purification Step. Even though wide variations have been made in the amount of exchanger used, a substantial portion (15–20%) of the activity is not held by the column, and repassage of this effluent through a second column does not result in further adsorption. Efforts have not been made to purify this nonadsorbed fraction.

Elution from IRC-50 has been done essentially batchwise, as described above. Attempts to elute slowly by means of a salt gradient have not revealed any discontinuities in the 280-mμ absorbancy curve or in the corresponding curve of bioactivity. Regardless of the rate of elution, however, it has been observed that the specific activity of the eluate is greater at the start of the process than at the end. As described under step 2 of the purification procedure, the eluate from

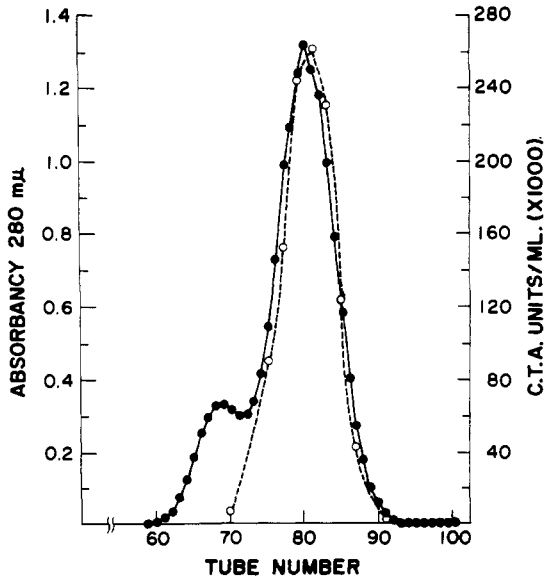

FIGURE 3: Gel filtration of step 5 S1 urokinase. A column 3.0 × 105 cm was prepared with Sephadex G-100 equilibrated *vs.* pH 6.5 buffer (0.1 M NaCl, 0.1 M phosphate). Sample (80 mg) was applied in 2.2 ml of buffer. Fraction volume was 4.6 ml/tube. A pool of tubes 79–87 was taken as the main fraction for further study as step 6 S1. This fraction contained 38 of the original 83 units of 280-mμ absorbancy applied to the column. ●——●, absorbancy; ○——○, bioactivity.

IRC-50 has been split into the main portion of the elution peak (fraction A) and the tail portion (fraction B). Purification of activity beyond step 2 has been done mainly with fraction A as described above. However, fraction B has also been carried through the gel filtration of step 3 and the hydroxylapatite columns of step 5. In gel filtration the pattern is substantially the same as that shown in Figure 1 for the main fraction. However, in chromatography on hydroxylapatite an active peak additional to those shown in Figure 2 emerges at a higher concentration of buffer. The two peaks of Figure 2 emerge before the buffer concentration reaches 0.15 M, while the additional peak emerges at a concentration >0.2 M. This fraction, designated step 5T, has shown the following properties: specific activity, 35,000–40,000 units/A_{280}, $s_{20,w}$ = 3.26, and an electrophoretic mobility at pH 4.8 in the range of 8×10^{-5} cm²/sec/v.

Analysis of Step 3 Fractions. Efforts have been made to characterize the inert peaks resulting from the step 3 gel filtration procedure. Peak A, which is excluded in Sephadex G-100, appears to be composed of high molecular weight glycoprotein material. Peak B has been rerun in G-100 gel filtration, resulting in a symmetrical elution pattern. The peak emerged earlier than human serum albumin, run separately as reference, suggesting that peak B is greater than 70,000 mol wt. Fraction B has been tested in immunoelectrophoresis *vs.* antiserum to human serum and two weak bands occurred, corresponding to haptoglobin and transferrin. The levels of these two substances were estimated to be no more than a few per cent, leaving the bulk of peak B unidentified. By contrast, the active C peak showed no cross-reaction with human serum antiserum. Both peaks A and B were highly pyrogenic and contained more thromboplastin impurities, measured by the method of Alkjaersig *et al.* (1965), than peak C. The low molecular weight of peak D as indicated by its late emergence from Sephadex G-100 and its $s_{20,w}$ value (2.1 S), as well as the reported occurrence of ribonuclease in urine (Delaney, 1963), suggested that peak D should be tested for enzymatic activity. When assayed *vs.* yeast nucleic acid by the method of McDonald (1955), peak D showed strong activity indicative of a ribonuclease content exceeding 50%.

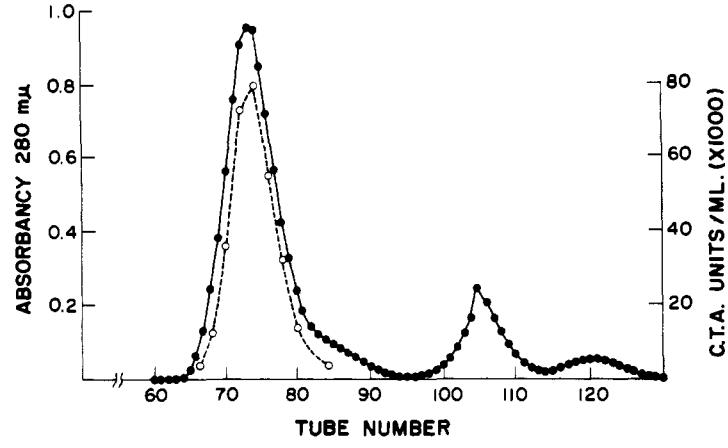

FIGURE 4: Gel filtration of step 5 S2 urokinase. Same column and conditions as in Figure 3. Sample (80 mg) on in 2.0 ml of buffer. Pool of tubes 69–79 taken as main fraction for further study as step 6 S2. This fraction contained 34 of the original 56 units of 280-mμ absorbancy applied to the column. ●——●, absorbancy; ○——○, bioactivity.

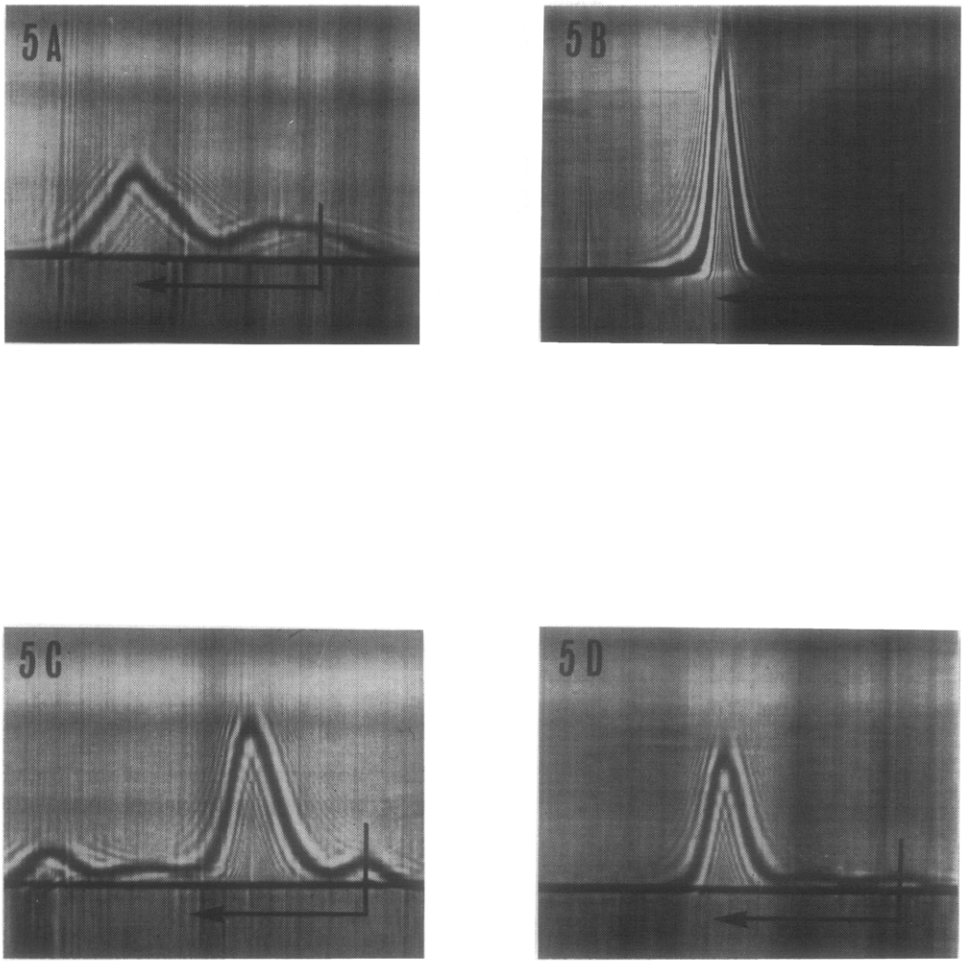

FIGURE 5: Moving-boundary electrophoresis of urokinase preparations. All patterns are at pH 4.8, acetate buffer, $\mu = 0.1$. Ascending limb shown; arrow gives direction of electrophoretic movement toward the cathode. 5A: Stage 5, S1, 58.7 min at 5.7 v/cm. 5B: Stage 6, S1, 64.3 min at 5.4 v/cm. 5C: Stage 5, S2, 65.4 min at 5.7 v/cm. 5D: Stage 6, S2, 51.1 min at 5.4 v/cm.

Final Separation of Two Main Types. A preliminary study of the physical and immunochemical properties of hydroxylapatite (step 5) fraction S1 and S2 (Figure 2) suggested a close approach to homogeneity. Further purification was accomplished by gel fitration in Sephadex G-100. As seen in Figures 3 and 4, the bioactivity is symmetrically related to the main A_{280} peaks. Portions of the peaks cut as shown in the legend were used for final physical and immunochemical characterization.

Electrophoresis. The purification of urokinase was followed by moving boundary electrophoresis, starting with step 2 material. The electrophoretic pattern for step 2 material at pH 4.8, ionic strength 0.1, in acetate buffer showed a pattern similar to that reported by Ploug and Kjeldgaard (1957) in their original work. The electrophoretic patterns continued to simplify until at step 6 essentially single boundaries were obtained. The electrophoretic patterns for both step 5 and 6 materials are shown in Figure 5. As can be seen, both S1 and S2 fractions at step 6 show substantially single components, whereas at step 5 both fractions showed multiple boundaries. Calculated mobilities for step 6 S1 and S2 fractions are shown in Table II.

Molecular Weight Determinations. Sedimentation analysis was a much less sensitive method for following the purification of the enzyme. Beginning with step 3C, single boundaries were observed for all active fractions, with $s_{20,w}$ values recorded in the range 2.7–3.0 S. After step 5 purification when two distinct active fractions S1 and S2 were separated, sedimentation coefficients of 2.66 and 3.30, respectively, were observed. Figure 6 shows the patterns obtained for the step 6 fractions. Molecular weight determinations were made on these step 6 fractions, the results being shown in Table II. By both sedimentation diffusion and sedimentation equilibrium the values for the S1 fraction are within experimental variation of an average calculated value of 31,500. The sedimentation equilibrium plot, as well as the boundary spreading measurements at three different pH values, showed that step 6 S1 fraction was paucidispersed. The molecular weight for the

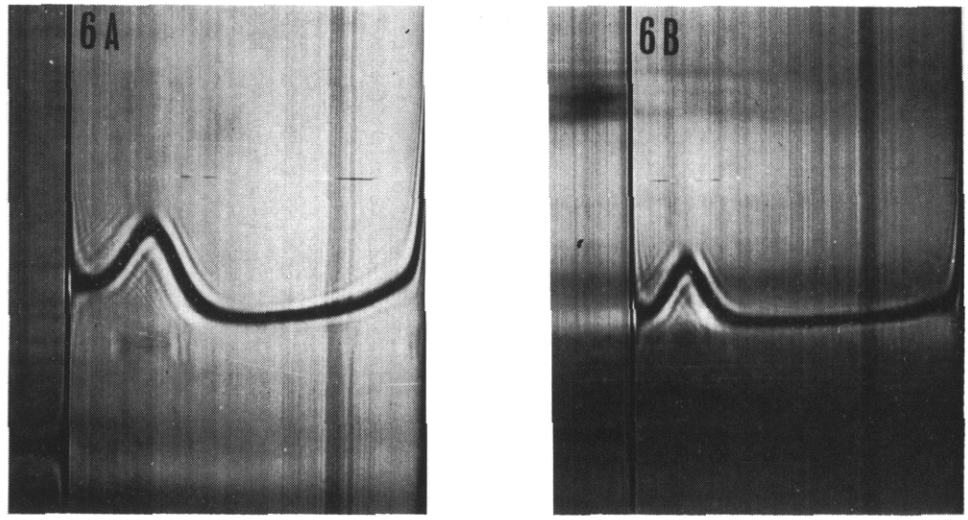

FIGURE 6: Sedimentation velocity patterns on urokinase preparations. Both photographs taken 64 min after attaining speed of 59, 780 rpm. 6A: Stage 6, S1. 6B: Stage 6, S2.

TABLE II: Physical Constants on Urokinase (Step 6).

	S1	S2
Sedimentation coefficient ($s_{20,w}$), S		
pH 2.6	...	3.18
pH 4.5	2.75	3.33
pH 6.5	2.66	3.27
pH 8.5	2.71	...
Heterogeneity constant (ρ)		
pH 4.5	0.28×10^{-13}	...
pH 6.5	0.35	...
pH 8.5	0.32	...
Diffusion coefficient ($D_{20,w}$), cm^2/sec		
pH 6.5	7.41×10^{-7}	...
Partial specific volume,[a] V, ml/g	0.724	0.728
Frictional ratio	1.35	...
Molecular weight		
S and D	31,700	...
Equilibrium	31,300	54,700
Electrophoretic mobility $\times 10^5$ cm^2/v sec (pH 4.8, acetate buffer, μ 0.1)	+3.5	+2.2
$E^{1\%}_{1cm}$, 280 mμ, pH 6.5	13.2	13.6

[a] Calculated from amino acid composition data (Schachman, 1957).

step 6 S2 fraction was determined only by sedimentation equilibrium and a value of 54,700 was obtained. Unlike step 6 S1, the S2 equilibrium plot showed polydispersity deviating from linearity both at the meniscus and at the bottom of the centrifuge cell.

Immunochemistry. Attempts were made to produce antisera to several fractions at step 3 and later stages of purification. After the initial immunization schedule for the active step 3C fraction, three main lines were observed between the antiserum and the immunizing antigen. By cross-reaction tests in double diffusion it was found that the strongest line corresponded to the inert adjacent fraction step 3B, rather than to the bioactive C fraction. Due to the presence of urokinase inhibitors (Von Kaulla, 1963) in all rabbit sera, it was necessary to separate and purify the γ-globulin fraction before testing the ability of the antibodies to neutralize fibrinolytic activity. The purified γ-globulin from the antiserum to step 3C fraction had the relatively low neutralization value of 1000 CTA units/ml or 500 CTA units/ml of original antiserum. The γ-globulin fraction from normal rabbit serum had no quenching effect on urokinase.

Two rabbits were immunized against step 5 S1 fraction. One animal responded rapidly with antisera which showed only two bands in double diffusion against the antigen, the more mobile band being markedly stronger than the other. Tests of serial dilution of the antigen in the Feinberg No. 1812 array (Feinberg, 1956) showed visible banding down to an antigen concentration of 0.032 mg/ml. This end point was attained within a week of the last immunizing injection and showed no change over a 2-month period. The antiserum and the γ-globulin fraction have been used to test the purity of the step 6 fractions. Figure 7A shows a double diffusion test in the Feinberg No. 1803 array. The top well contains the immunizing step 5 S1 antigen and shows the two bands *vs.* both raw antiserum (right) and the γ-globulin solution (left). By contrast, the highly purified step 6 S1 fraction in the bottom well forms only one band in each case.

The γ-globulin solution from the antiserum to step 5 S1 gave a neutralization value of 40,000 CTA units/ml, or 20,000 CTA units/ml of original antiserum.

As shown in Figure 7B, the two highly purified step 6 fractions showed the reactions of partial identity in double diffusion *vs.* the high titer antiserum to step 5 S1 fraction. It will be noted that the band to S2 is much less intense than the band to S1.

Fibrinolytic and Esterolytic Activities of Step 6 Fractions. By the fibrinolytic assay, fractions step 6 S1 and step 6 S2 showed values of 218,000 and 93,500 CTA units/mg of protein, respectively. Step 6 S1 fraction was also assayed by the acetyl-L-lysine methyl ester (ALMe) assay (Sherry *et al.*, 1964). By measurement of methanol release, the specific activity of step 6 S1 was found to be 0.69×10^{-3} μmole/min/CTA unit. The CTA standard preparation run at the same time as a reference gave a value of 0.88×10^{-3} μmole/min/unit. Using this value for the standard the specific activity of step 6-S1 is 170,000 CTA units/mg of protein. This value was somewhat higher than the figure of 0.77×10^{-3} μmole/min/unit used by Sherry *et al.* in defining the CTA unit.

Optical Absorbancy of Step 6 Fractions. Optical absorbancy was measured in the pH 6.5 buffer. $E^{1\%}_{1\,cm}$ was calculated from the 280-mμ reading using a protein value obtained by calculation from the total nitrogen determination. The results are shown in Table II.

Amino Acid Composition of the Step 6 Fractions. Table III shows the results of amino acid composition analyses of step 6 S1 and S2 fractions. Because of the small amount of starting material used in these analyses, corrections for ash, moisture, and carbohydrate were not made. This presumably accounts for the fact that the total recovery of amino acids is <100%.

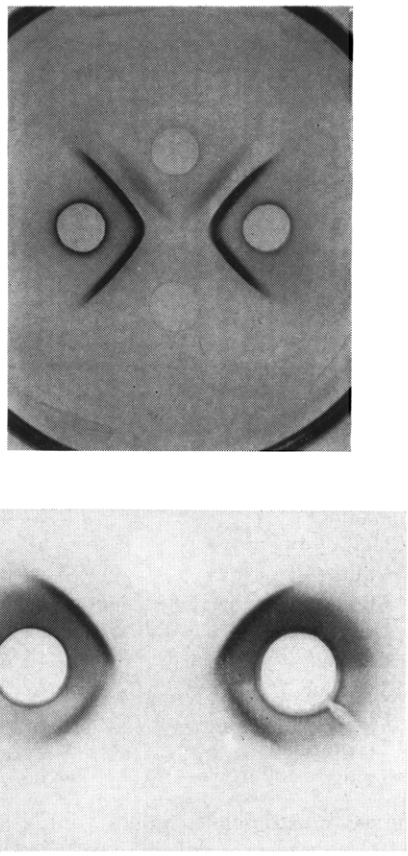

FIGURE 7: Immunodiffusion patterns. (A) Comparison of step 5 S1 (top) with step 6 S1 (bottom), using antiserum (right well) and γ-globulins (left well) to fraction step 5 S1. (B) Demonstration of reaction of identity between step 6 S1 (top) and step 6 S2 (bottom). In all cases, the antigen solutions were used at 1 mg/ml.

Discussion

The reasons for the discrepancies between our ultracentrifugation results and those reported by Lesuk *et al.* (1965) is not clear. They reported differences in boundary spreading as a function of pH, whereas our S1 fraction when subjected to boundary spreading measurements (Table II) showed no pH dependence, and would therefore indicate no association phenomenon occurring at any pH value. Since Lesuk *et al.* showed no comparable quantitative spreading data it is difficult to assess the amount of spreading involved. A second discrepancy involves differences in molecular weight for our principal material (31,600) and their crystalline material (54,000). Even though the Archibald method used by the latter investigators presents problems in the determination of $(dc/dx)_m$ (Ginsburg *et al.*, 1956) which sometimes results in high estimations of molecular weight (Keresztes-Nagy and Klotz, 1963), it is difficult to believe that this could be responsible for such a large difference in values. Again, it seems unlikely that urokinase exists naturally in the form of a large molecular weight complex since Burges *et al.* (1965), using a gel filtration technique with a crude sample of urokinase and employing bioactivity as monitor, found a molecular weight value (34,500) very close to our own. Thus, it appears most likely that the value obtained by Lesuk *et al.* was for an artifactual complex of urokinase with an inert substance, presumably protein, which was not eliminated by the crystallization procedure. This is further supported by the twofold greater specific activity of our S1 fraction (218,000 units/mg) compared to their crystalline material (104,000 units/mg).

We have considered the possibility that our type S2 is the result of an association of type S1 with an inert protein of roughly the same size. However, in our manipulations of the S2 form, we have not observed any dissociation into smaller molecules. Indeed, we have attempted to break up type S2 by treatment with succinic anhydride (Keresztes-Nagy and Klotz, 1963) without success. The resulting modified protein did not show any significant change in molecular weight by sedimentation equilibrium nor did the unmodified protein show changes in sedimentation observed under conditions of widely varying pH (Table II) or high concentrations of urea (6 M).

TABLE III: Amino Acid Composition of Urokinase Fractions Step 6, S1, and S2.

	Amino Acid Residues (g/16 g of Protein N)[a]		Amino Acid Residues per	
	S1	S2	31,500 g of Protein (S1)[d]	54,700 g of Protein (S2)[d]
Lysine	7.04 ± 0.23[e]	7.27 ± 0.15[e]	17.3	31.0
Histidine	4.44 ± 0.34	4.94 ± 0.20	10.2	19.7
Arginine	7.04 ± 0.25	6.31 ± 0.45	14.3	22.1
Aspartic acid	6.96 ± 0.26	8.52 ± 0.08	19.1	40.4
Threonine[c]	6.08 ± 0.14	5.87 ± 0.27	19.0	31.6
Serine[c]	6.01 ± 0.08	5.56 ± 0.12	21.8	34.8
Glutamic acid	11.56 ± 0.40	10.42 ± 0.28	28.3	44.1
Proline	5.04 ± 0.19	5.10 ± 0.23	16.4	28.7
Glycine	4.04 ± 0.16	4.29 ± 0.07	22.4	41.1
Alanine	2.27 ± 0.13	2.63 ± 0.08	10.1	20.2
Half-cystine	3.10 ± 0.14	3.68 ± 0.42	9.5	19.4
Valine[f]	3.42 ± 0.24	4.21 ± 0.06	10.9	23.2
Methionine	1.85 ± 0.09	1.85 ± 0.09	4.5	7.7
Isoleucine[f]	5.58 ± 0.09	4.25 ± 0.04	15.6	20.4
Leucine	7.35 ± 0.24	6.95 ± 0.05	20.6	33.6
Tyrosine	6.62 ± 0.37	5.96 ± 0.16	12.8	19.9
Phenylalanine	4.32 ± 0.60	3.86 ± 0.42	9.3	14.3
Tryptophan[b]	2.91	3.27	4.9	9.6
Totals	94.63	94.94		

[a] Average or extrapolated values from 22–72-hr hydrolysates. [b] Estimated on intact protein. [c] Value extropolated to zero time. [d] Assume protein to be 16% nitrogen. [e] Average deviation. [f] Value from 72-hr hydrolysate only.

Because of the fact that type S2 has only about one-half of the activity of S1 toward plasminogen and toward antiserum to type S1, we are continuing our efforts to dissociate the S2 molecule. The amino acid compositions of the two types (Table III) show a marked similarity, but they do not exclude the possibility that the S2 molecule is made up by the association of S1 with an inert protein of approximately the same size and composition.

Because of the limitation in material, free electrophoresis was performed at one pH only. The direction of movement toward the cathode at pH 4.8 indicated an isoelectric point above that pH for both of the major forms of urokinase. In the case of type S1, an attempt was made to determine the isoionic point by the use of the micro apparatus of Katz and Ellinger (1963). After a preliminary period during which the amperage dropped to 7 ma a sharp fall in pH was observed to a limiting value of 2.5. During the sharp fall in pH, an irreversible precipitation of protein was observed. The same tendency toward insolubilization of urokinase has been observed during prolonged dialysis, although no extremes of pH have accompanied it. We are able to account for these phenomena only by the postulation of a strong negative charge on type S1 urokinase, resulting in an unusually strong binding of cations.

We have speculated on the origin of the minor types of urokinase which have been observed in the course of purification, but on which little work has been done because of the relative scarcity of material. Type T, which originates in the fraction from the late stages of elution from IRC-50 resin and is separated from the two main types on hydroxylapatite, has approximately the same sedimentation value as type S2, but is more basic. Since it does not appear likely that it is derived from either of the two main types by degradation, possibly it is a genetic variant.

On the other hand, the C-2 type (Figure 1) is clearly of smaller molecular size than either of the purified types derived from Sephadex peak C-1. Thus, the possibility of proteolytic degradation must be considered. The occurrence of pepsin-like (uropepsin) activity in urine has been reported frequently (Von Kaulla, 1963). Since Mirsky *et al.* (1948) have shown that uropepsin is derived by internal secretion from the same stomach cells and zymogen which give rise to pepsin by external secretion, we have tested the effect of gastric pepsin (hog) on urokinase at pH values from the low values optimal for pepsin to the pH of urine. Urokinase is very susceptible to peptic action at pH 2.5, being completely degraded and inactivated by traces of pepsin in a matter of minutes. At pH 5.2 with pepsin in a ratio of 0.1% by weight to urokinase, bioactivity is lost slowly over 24 hr with only moderate evidence of proteolytic action. Thus, it appears possible that exposure of a larger molecule such as type S1

urokinase to the action of pepsin during storage of urine in the bladder could give rise to a smaller fragment of lower specific activity. The possibility of accomplishing this conversion using purified urokinase and crystalline pepsin *in vitro* is being explored.

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