# Purification and Characterization of Streptokinase with Studies of Streptokinase Activation of Plasminogen*

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ABSTRACT: Streptokinase was chromatographed successively on DEAE Sephadex A-50 (0.01 M Tris–HCl buffer, pH 8.5, with a linear gradient 0.1–0.5 M NaCl at 4°) and on Sephadex G-100 (0.001 M phosphate–0.3 M NaCl, pH 7.4, at 4°). Specific activity was increased 11-fold and was associated with separation of impurities which migrated in the $\alpha_2$, $\alpha_1$, $\beta_1$, $\beta_2$, and $\gamma$ regions. Streptokinase was identified as an $\alpha_1$-globulin. Physical chemical analysis revealed an extinction coefficient ($E^{1\%}_{1\,cm}$) of 9.49 at 280 m$\mu$, specific viscosity of 0.10, partial specific volume of 0.738 ml/g, $s_{20,w}$ of 3.1 S, and a molecular weight estimated by sedimentation equilibrium and gel filtration techniques of 50,000 ± 3000. Chemical analysis revealed a low hexose content of 0.2 g %, hexosamine of 0.1 g %, and nitrogen of 14.8 g %. Amino acid analysis revealed no methionine or cysteine residues. Optical rotatory dispersion studies suggest that 12% of the molecule is in the form of a helix. Study of the interaction of this purified streptokinase with purified plasminogen (prepared from Cutter pseudoglobulin plasminogen lot 660-40) revealed in eight out of ten studies a split product of a molecular weight of 6200 ± 1000) which originated from *plasminogen*. This product appeared within 5 min and it constituted 15% of the starting material. Neither the specific activity of the plasmin nor the amount of the split product varied over the 20–30 min (at 22°) following the initial 5-min activation period. Therefore, we concluded that this split product was associated with some phase of plasminogen conversion to plasmin. No split products originating from *streptokinase* within the first 5 min were detected. However, split products amounting to 1–3% of the starting material were observed within 20 min at 22°. The hydrogen ion release accompanying this reaction was studied with the pH-Stat and hydrogen ion equivalent to the hydrolysis of only one to two peptide bonds was released.

The nature of the streptokinase activation of plasminogen has been the subject of intense study over the last 10 years because of the unique effect of streptokinase (SK)[1] on the activities arising from plasminogen (*i.e.*, plasmin and activator activities).

Evidence has been presented by Kline and Fishman (1961) that streptokinase reacts directly with plasmin to form a complex with increased affinity for certain synthetic substrates (*e.g.*, lysine methyl ester) and decreased affinity for certain protein substrates (*e.g.*, casein). This plasmin–SK complex was termed "activator" because: (1) it was an efficient activator of bovine plasminogen whereas SK or plasmin alone was not, and (2) it was an effective activator of human plasminogen whereas plasmin alone was not. From this and other evidence it was assumed that SK activation of plasminogen is mediated through SK reaction with the small amount of plasmin (≈1%) which is always associated with plasminogen to form the activator which, in turn, initiates the further conversion of plasminogen to plasmin (Kline and Fishman, 1961). The possible existence of the plasmin–SK activator complex has also been supported by Davis *et al.* (1964), who showed that a 1:1 molar ratio of streptokinase to plasmin (or plasminogen) formed a complex which sedimented faster in the ultracentrifuge than either of the two components alone and which had activator activity.

Though the existence of such a plasmin–SK complex appears to have been established, little is known about the further activation of plasminogen. For example, it is not clearly established whether any split products are formed during the formation of the plasmin–SK complex and subsequent activation of plasminogen, nor has it been demonstrated directly whether SK activates plasminogen through modification of plasmin activity. These questions have remained unanswered, in part because of a lack of highly purified streptokinase which is necessary for such studies to be undertaken.

This communication presents (1) a method for purifying streptokinase and some of the characteristics of this highly purified material, and (2) an investigation of the interaction of SK and plasminogen including the nature and source of the split product arising from this reaction.

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[1] Abbreviations used: SK, streptokinase; LME, lysine methyl ester; EACA, ε-aminocaproic acid.

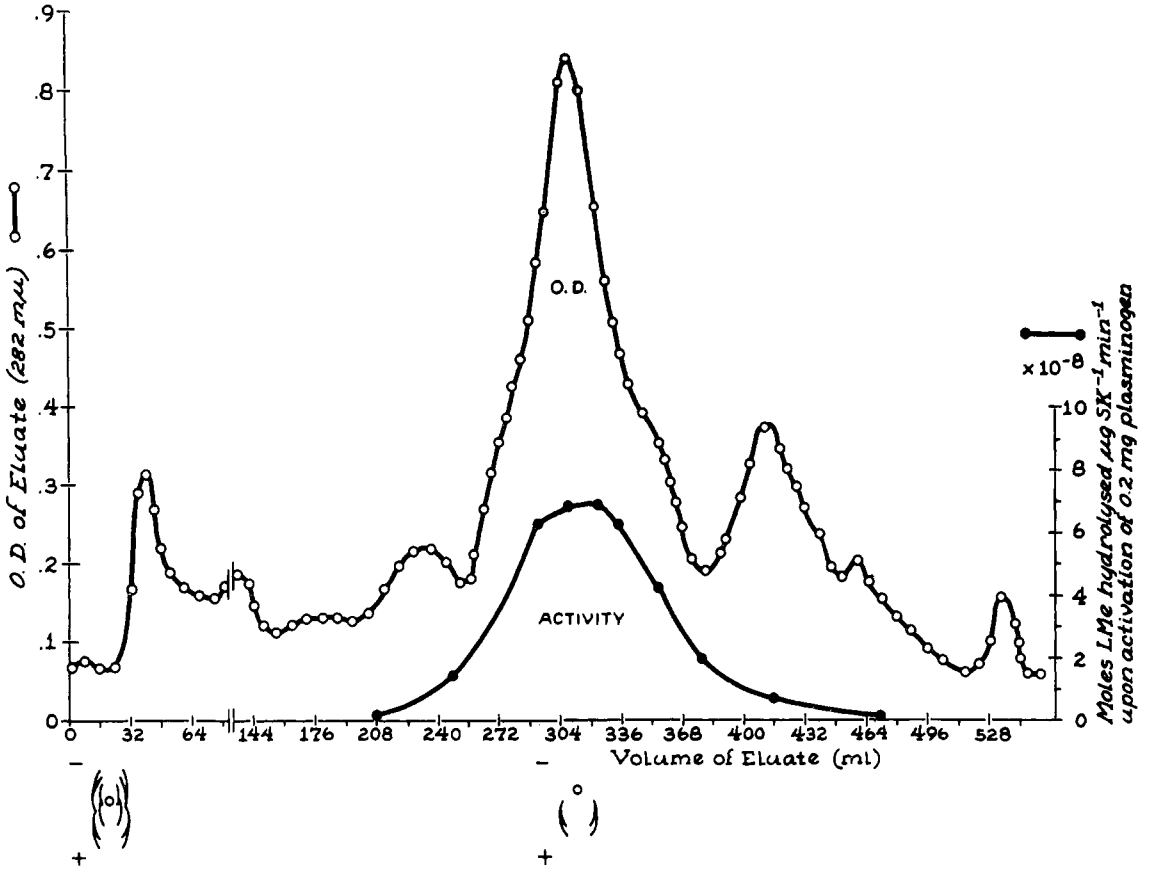


FIGURE 1: Elution pattern (○—○) and streptokinase activity (●—●) of streptokinase chromatographed in DEAE Sephadex A-50 in 0.01 Tris buffer at pH 8.5, 4°. Elution gradient was linear starting at an ionic strength of 0.10 and finishing at an ionic strength of 0.50. Immunoelectrophoretic patterns of the starting material and material containing streptokinase activity are also shown.

## Materials and Methods

*Preparation of Plasminogen.* Purified plasminogen was prepared from partially purified pseudoglobulin plasminogen (Cutter Laboratory lot 660-40) according to the method of Taylor and Staprans (1966). The specific activity of this purified plasminogen varied from 145 to 155 casein units per mg of protein nitrogen. This material was homogeneous by chromatographic and immunoelectrophoretic criteria. Aliquots (2 ml) of this plasminogen (40 mg/ml) were stored at −60° after dialysis for 48 hr at 4° against 2 l. of 0.30 M NaCl and 0.03 M phosphate buffer (pH 7.4). The plasminogen used for pH-Stat studies was dialyzed for 48 hr at 4° against 2 l. of 0.30 M NaCl alone and was then used immediately.

*Preparation of Plasmin.* Plasmin was prepared from purified plasminogen (20 mg) by activation with 400 units of urokinase (lot 752–8945) which was obtained from Abbot Laboratories, North Chicago, Ill., and used within 2 hr. Plasmin activity was monitored with the assay described below.

### *Preparation and Characterization of Streptokinase*

*Fractionation of Streptokinase.* Purified streptokinase was prepared from Varidase (lot 108–478) kindly provided by Lederle Laboratories, Fort Washington, Pa. This material was further purified in the following manner. Approximately 40 mg of SK was dissolved in 3 ml of 0.10 M NaCl–0.01 M Tris buffer (pH 8.5) and dialyzed for 48 hr at 4° against two changes of 2-l. volumes of the same buffer to remove chromagens. This material was then adsorbed and fractionated on DEAE Sephadex A-50 at 4° employing a linear NaCl–Tris gradient (Figure 1). The DEAE Sephadex A-50 was obtained from Pharmacia Fine Co., Uppsala, Sweden. This material had a binding capacity of 3.5 ± 0.5 mequiv/g. Lots (5–10 g) of the DEAE Sephadex were washed eight times with 1-l. volumes of 0.1 M NaCl–0.01 M Tris buffer (pH 8.5) and allowed to equilibrate with this buffer over 3 days after a final adjustment of the pH of the slurry to pH 8.5 with 0.1 N HCl. This was packed into a 45 × 3 cm column by gravity (at a flow rate of 20 ml/hr). The total bed

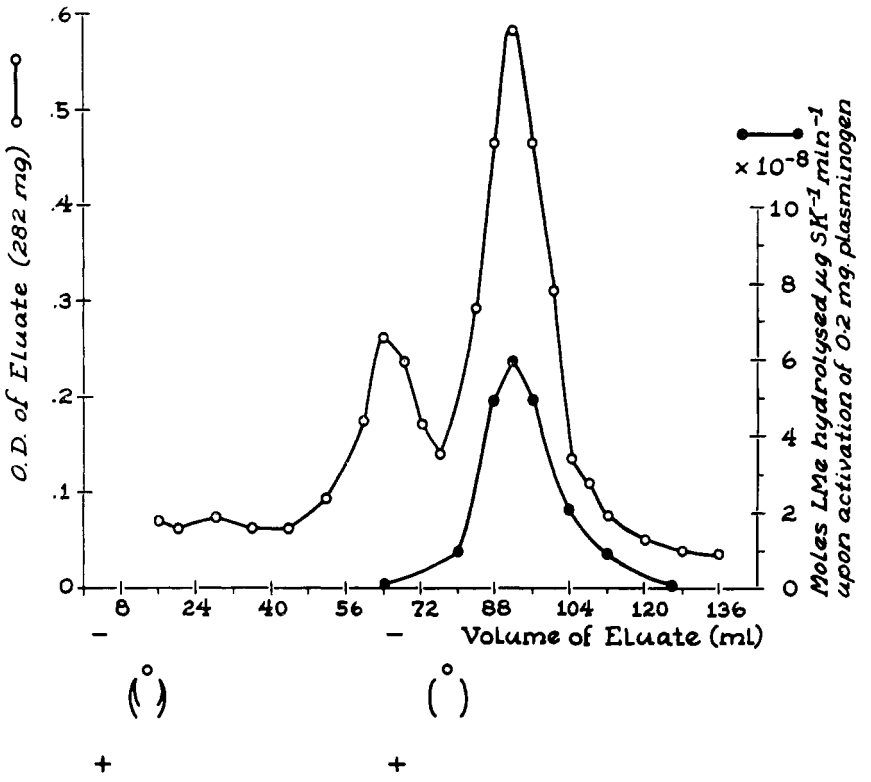


FIGURE 2: Elution pattern (○—○) and streptokinase activity (●—●) of the streptokinase eluate from Sephadex A-50 filtered through Sephadex G-100 in 0.001 M phosphate–0.3 M NaCl buffer solution at pH 7.4, 4°. Immunoelectrophoretic patterns of the starting material and material containing streptokinase activity are also shown.

volume was 250 ml. The linear gradient was formed by using two 300-ml erlenmeyer flasks (level with each other and open to the atmosphere) that were connected in series to each other and the column. These two flasks were connected at their bases by a capillary tube and hence to the column by a $^1/_8$-in. diameter polyethylene tube and plug. The first flask contained 250 ml of a 0.5 M NaCl–0.01 M Tris solution that ran into the second flask (mixing flask) which contained 0.1 M NaCl–0.01 M Tris. The thoroughly mixed solutions were then delivered into the top of the column by gravity feed at a rate of 20 ml/hr. The total elution volume was 500 ml collected in 2–3-ml aliquots by a GME Model T 15[2] fraction collector. The protein concentration was determined by absorbance measurements in 1-cm quartz cells at 280 mμ with a Beckman DB spectrophotometer. The effluent containing SK was pooled and dialyzed for 48 hr at 4° against three changes of 2-l. volumes of 0.001 M phosphate and 0.3 M NaCl at pH 7.4. This dialysate was then fractionated on G-100 Sephadex gel at 4° at a flow rate of 20 ml/hr (Figure 2). The Sephadex G-100 was supplied by Pharmacia Fine Co., Uppsala, Sweden. The dry powder was suspended in a sufficient amount of phosphate buffer (0.001 M phosphate–0.30 M NaCl at pH 7.4) and stored for 72 hr to assure complete swelling. The fines were removed by several decantations and the gel was packed into a 45 × 3 cm column. The total bed volume was 250 ml and the void volume was 32 ml as determined with Blue Dextran. The streptokinase was pooled, concentrated by ultrafiltration, and stored in 2-ml volumes at −60°.

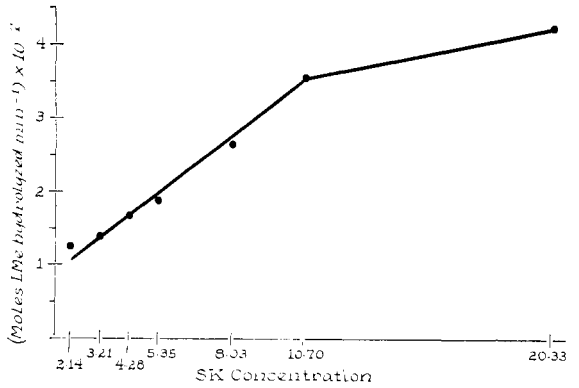


FIGURE 3: Standard curve for moles of LMe hydrolyzed per unit time by 0.2 mg of plasminogen as a function of streptokinase concentration (μg/ml).

*Assay of Streptokinase Activity.* The SK activity of the eluates described above was monitored indirectly by assaying the amount of plasminogen converted to plasmin by SK employing the lysine methyl ester

(LMe) esterolytic assay described by Roberts (1962). In this assay, 0.200 mg of plasminogen (0.25 ml) was activated by 0.25 ml of the SK sample to be tested with 0.5 ml of 0.08 M LMe in Tris buffer (pH 7.5), 37°. The LMe was supplied by Mann Research Co., New York, N. Y.

In defining the activity of SK, it is necessary to do so indirectly in terms of the amount of plasmin activity on the LMe generated by the SK conversion of plasminogen to plasmin. Therefore, for each given batch or lot of SK, we established a standard curve of moles of LMe hydrolyzed per minute by 0.200 mg of plasmin *vs.* the concentration of SK in micrograms per milliliter of assay solution (Figure 3). The specific activity of the plasmin used had to be constant from assay to assay and this was 80 Remmert and Cohen units/mg of nitrogen. In order that the activity of different lots of SK could be compared under the conditions described above, SK activity was expressed in units. One unit of SK activity was arbitrarily defined as $1 \times 10^{-9}$ mole of LMe hydrolyzed $min^{-1}$ mg of $N^{-1}$ by 0.200 mg of plasmin as in eq 1.

$$\frac{\text{(units of SK activity)}}{\text{mg of N of SK}} = \frac{(\Delta\text{OD during 30-min reaction})(\epsilon\ 2.79 \times 10^{-8})(10^3)}{(30\text{ min})(\text{number of mg of N})(1 \times 10^{-9})} \quad (1)$$

Aliquots (2 ml) (10 mg/ml) of the purified SK prepared and assayed as described above were stored at −60° after dialysis for 48 hr at 4° against 2 l. of 0.001 M phosphate–0.30 M NaCl (pH 7.4). In the case of SK to be used in the pH-Stat studies, dialysis of the sample was carried out at 4° for 12 hr against two changes of 2-l. volumes of 0.3 M NaCl immediately before use.

*Preparation of Antisera to Streptokinase.* Antisera to plasminogen and SK were prepared as described previously and stored at 4° in 0.01% merthiolate (Taylor and Staprans, 1966). Immunoelectrophoresis in agar gel was also performed as described previously using optimal concentrations of SK (0.5–1 mg/0.1 ml) for production of precipitation lines (Taylor and Staprans, 1966). The diffusion patterns were developed at 22° for 24 hr. The slides were washed, dried, and stained with Amido Black.

*Extinction Coefficient.* The extinction coefficient at 280 mμ of streptokinase was calculated from measurements in 0.1 N NaOH after subtraction of absorption at 320 mμ and was corrected for moisture content.

*Optical Rotatory Dispersion.* Optical rotation measurements of 0.037% solutions of streptokinase were made with a Durrum–Jasco automatic recording spectropolarimeter, Model ORD/UV-5, using a xenon arc light source. A 0.05-dm cell was used and the material was examined at wavelengths from 220 to 600 mμ.

The optical rotatory dispersion data were evaluated in terms of the magnitude of the rotatory trough at 233 mμ as expressed by Simmons *et al.* (1961) in the following formula

$$R = \left[\frac{100(\alpha\lambda)}{(l)(c)}\right]\left[\frac{\bar{M}}{100}\,\frac{3}{n^2+2}\right]$$

where $\alpha$ is the observed rotation in degrees at wavelength $\lambda$, $l$ is the cell length in decimeters, $c$ is the concentration of solute in g/100 ml, $\bar{M}$ is the mean amino acid residue weight (used here at 115), and $n$ is the index of refraction of the solvent. The $n$ value was taken from the compilations of Timmermans (1960) and evaluated as 1.395 at 233 mμ. The per cent helix was calculated from the depth of the trough of the Cotton effect assuming that the residue rotation at 233 mμ of a fully coiled helix is −16,200° and a randomly coiled structure is −1700° with a linear interpolation (Jirgenson, 1966).

*Viscosity.* The viscosities at 37 ± 0.01° of 1% solutions of streptokinase in 0.001 M phosphate buffer and 0.1 M NaCl (pH 7.4) and of the solvents relative to water were determined with an Ostwald-type viscometer which had an outflow time of 70 sec at 37° for water.

*Density.* The densities of 1% solutions of streptokinase and of the solvents relative to water were determined with a 5- and 50-ml pycometer at 22 ± 0.5°.

*Partial Specific Volume.* A partial specific volume of 0.738 was calculated from density measurements and from amino acid composition data employing the following equations, respectively

$$\bar{V}_{app} = \left[\frac{1}{d_0} - \frac{1}{x}\right]\left[\frac{d - d_0}{d_0}\right]$$

where $d$ and $d_0$ are densities of the solution and solvent, respectively, and $x$ is the concentration of protein in grams per milliliter of solution.

$$\bar{V} = \frac{\text{volume per cent of AA residues}}{\text{weight per cent of AA residues}}$$

This figure was used in the calculation of $s_{20,w}$ and molecular weight estimated from sedimentation equilibrium studies.

*Sedimentation velocity* and equilibrium experiments on 0.05, 0.075, 0.1, and 0.33% solutions of streptokinase in 0.1 M phosphate buffer–0.1 M NaCl were performed at 20° in a Spinco Model E analytical ultracentrifuge equipped with a phase plate, schlieren diaphragm, and rotor temperature indicator and control unit. Regular standard sectorial cells with a 1.2-cm light path were used and the temperature was maintained at 20 ± 0.2°. The sedimentation coefficients were determined from experiments in which the rotor was operated at 59,780 rpm. The positions of the schlieren peaks on the photographic plates were read directly with a micrometer and the sedimentation coefficients were calculated from the slope of the usual semilog plot. The observed values were then corrected to standard conditions of water at 20°. Correction for the effect of temperature on the viscosity of water was calculated from data in the Handbook of Chemistry and Physics (1965).

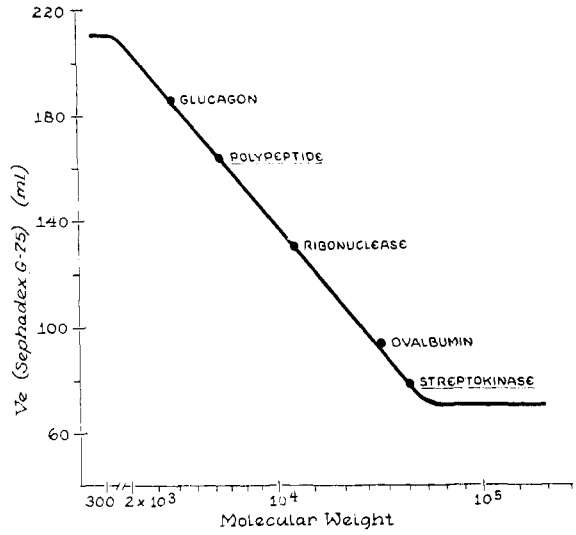


FIGURE 4: Plots of elution volumes ($v_e$) against log (molecular weight) for glucagon, ribonuclease, ovalbumin, and the polypeptide and streptokinase unknowns on a Sephadex G-75 column (3.0 × 36 cm equilibrated with 0.05 M Tris-HCl chloride buffer containing 0.1 *m* KCl, pH 7.5).

*Approach to equilibrium* determinations of 0.05, 0.075, 0.1, and 0.33% solutions of streptokinase in 0.001 M phosphate–0.1 M NaCl was determined with a standard cell at 20°, 120 min after a speed of 11,280 rpm was reached. The pattern was traced from the enlarged image of the photographic plate. These were done in triplicate for the pattern at both the meniscus and bottom of the cell and the calculations were performed.

The patterns of 0.05, 0.075, 0.1, and 0.33% solutions of streptokinase (from which the area under the curve values ($C_0$) were calculated) were produced by overlaying the solution of protein with buffer through use of the synthetic boundary cell centrifuged at 11,280 rpm for 30 min. The patterns were traced from the enlarged image of the photographic plate. These were done in triplicate and the area under each of the curves (taken at the same bar angle as the approach to equilibrium pattern for each given protein (SK) concentration) was determined by use of a planimeter. The reproducibility of the method on our hands for the molecular weight range 60,000–80,000 was within 7%.

*Molecular Weight Estimation by Gel Filtration.* Estimations of the molecular weights of streptokinase and of the split product from plasminogen were made using G-75 Sephadex according to the method described by Andrews (1964). Glycogen (molecular weight 3000) (0.2 mg) obtained from Eli Lilly, four-times-crystallized ribonuclease from ox pancreas (molecular weight 17,000) (1 mg) obtained from Worthington Biochemical Corp., and five-times-crystallized ovalbumin (molecular weight 66,000) (1 mg) obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, were used to calibrate the column. The molecular weights of streptokinase and the split product from plasminogen were estimated directly from the linear portion of the curve describing the relationship between $V_e$ (exclusion volume) and the molecular weight of the above-named calibration proteins (Figure 4).

*Amino acid analysis* was performed according to published procedure (Moore *et al.*, 1958) in which aliquots of 1.5–2.5 mg of protein dissolved in glass-distilled constant-boiling HCl were hydrolyzed at 110° for 24 hr. The hydrolysates were colorless and water clear. The amino acid composition of each sample was determined with a Spinco Model 120 amino acid analyzer.

*Protein Nitrogen.* Total nitrogen was determined by the micro-Kjeldahl method (Willits *et al.*, 1949) after extensive dialysis of samples.

*Carbohydrate.* Hexose and hexosamine content was estimated by the methods described by Dische (1955a,b) and in Kabat and Mayer (1961), respectively.

*Streptokinase-Plasminogen Interaction*

*Products Arising from the Interaction of an Excess of Plasminogen with Streptokinase.* The split products arising from 10 to 50 mg of plasminogen reacting with 0.250–0.500 mg of streptokinase were separated by gel filtration on Sephadex G-100 which separated plasminogen (molecular weight 87,000) and streptokinase (molecular weight 50,000) from any split products (molecular weight less than 15,000) arising from streptokinase-induced hydrolysis of the large excess of plasminogen. The materials were allowed to react for varying time intervals (5, 10, 15, and 20 min) at 22° in a 4-ml volume of 0.001 M phosphate–0.3 M NaCl solution (pH 7.4). The reaction was stopped with 150 mg of ε-aminocaproic acid (0.5 M EACA) (obtained from Mann Research Co., New York, N. Y.). This 4-ml mixture was then fractionated at 4° on Sephadex G-100 which had been equilibrated for 3 days with a 0.001 M phosphate–0.3 M NaCl–0.5 M EACA solution. The Sephadex void volume was 25 ml and the elution was carried out at a rate of 20 ml/hr. The control samples consisted of plasminogen alone (10–40 mg), streptokinase alone (0.250–0.500 mg), and a mixture of these two reactants to which 0.5 M EACA had been added *before* mixing. Aliquots (2 ml) of the eluate were assayed for plasminogen and streptokinase activity and for protein by spectrophotometry at 280 mμ.

*Products Arising from the Interaction of an Excess of Streptokinase with Plasminogen.* An examination for split products arising from 10 to 40 mg of streptokinase reacting with 0.250–0.500 mg of plasminogen was conducted by gel filtration on Sephadex G-100 and subjected to the same procedures and analysis at the same time intervals as described in the preceding section. In those particular studies where no split products were detectable by spectrophotometry and where it was suspected that there might be small polypeptide or amino acid split products, an examination for α-amino acids of digested and undigested samples was made using the ninhydrin method de-

scribed by Kabat and Mayer (1961). This method as used will detect as little as 1 $\mu$g of amino acid nitrogen and therefore was considered capable of detecting as little as one amino acid split product arising from 40 to 50 mg of purified plasminogen or streptokinase.

*Release of Hydrogen Ions Accompanying the Interaction of Streptokinase with Plasminogen.* An attempt to estimate the number of peptide bonds hydrolyzed was made by monitoring the total amount of hydrogen ion released upon interaction of streptokinase and plasminogen with a pH-Stat (Radiometer, Copenhagen) at pH 7.58, 23°, under a nitrogen atmosphere with 0.004 M NaOH as the titrant. Three determinations were made using 65 mg of plasminogen ($7.50 \times 10^{-7}$ mole based on an estimated molecular weight of 87,000) and 17.16 mg of streptokinase ($2.83 \times 10^{-7}$ mole based on an estimated molecular weight of 50,000) in a total volume of 11 ml. The above reactants were dissolved in a 0.30 M NaCl solution with no buffer. The plasminogen solution (9 ml) was introduced into the reaction flask and the system was stabilized at pH 7.58, 23°. At the same time, the pH of the streptokinase solution was similarly stabilized at pH 7.58 prior to its addition of the plasminogen solution. Thus, upon addition of the streptokinase to the plasminogen solution the reaction volume was raised from 9 to 11 ml with no shift in pH in excess of 0.05 pH unit. The reaction was then titrated until no detectable hydrogen ion was released. The number of moles of hydrogen ion released in this system was calculated from the molarity of the base titrant and the volume introduced into the system by the calibrated syringe during the course of the reaction correcting for protonation of newly formed N-terminal amino acids.[2]

## Results

The activity of the streptokinase starting material (Varidase, lot 108–478) was $3.54 \times 10^4$ units/mg of N. This was increased 5.2 times to $18.4 \times 10^4$ units/mg of N after fractionation on DEAE on Sephadex A-50 and another 2.2 times to $39.4 \times 10^4$ units/mg of N after fractionation on Sephadex G-100. (On occasion it was necessary to rechromatograph the streptokinase on DEAE Sephadex before going on to the gel filtration step.) The increase in activity of various streptokinase preparations upon purification ranged from 6- to 11-fold. In this example the fractionation on DEAE Sephadex and Sephadex G-100 gave an 11-fold increase in activity or an increase in terms of Christensen units of 20,000–240,000 units.[3] The streptokinase on which physical chemical and chemical studies were done was recovered from the trailing half of peak two of the Sephadex G-100 fractionation. Rechromatography of this portion of peak two gave a homogeneous pattern. The fractionation of streptokinase as monitored by immunoelectrophoresis resulted in the elimination of at least five distinct contaminants, leaving one band in the $\alpha_1$-globulin region which was identified as SK.

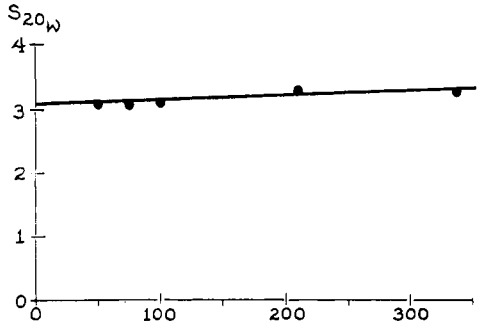


FIGURE 5: Graph of $s_{20,w}$ values *vs.* concentration of streptokinase in mg/100 ml. All values are corrected to standard conditions of water at 20°.

The extinction coefficient ($E_{1\,cm}^{1\%}$) at 280 m$\mu$ of purified streptokinase after correction for moisture content was 9.49. The average specific viscosity of streptokinase was 0.10 with values of the seven determinations ranging from 0.09 to 0.11. The per cent helix content of streptokinase as determined from the depth of the trough of the Cotton effect was 10–12%. The average partial specific volume of streptokinase in 0.001 M phosphate–0.1 M NaCl solution was 0.738 ml/g. The partial specific volumes ranged from 0.73 to 0.75 ml/g. The average value (above) was used in calculation of the $s_{20,w}$ values from the sedimentation velocity data and of molecular weight from the sedimentation equilibrium data, the estimate of which correlated closely with molecular weight estimated from the gel filtration studies. The partial specific volume as calculated from the amino acid data was 0.730 ml/g.

The sedimentation velocity of streptokinase varied slightly with the concentration of this material analyzed. Extrapolation of the $s_{20,w}$ values obtained from these experiments gave a sedimentation coefficient for streptokinase at infinite dilutions of 3.10 S (Figures 5 and 6).

The molecular weight as estimated from four approach to equilibrium runs at each of four different concentrations was 50,000 ± 3000 (Figure 7). Estimation of molecular weight employing the gel filtration method of Andrews repeatedly yielded a molecular weight value of 55,000 ± 2000 for streptokinase (Figure 4).

Amino acid analysis of streptokinase revealed a high proportion of glutamic and aspartic acids with no methionine or cysteine residues (Table I).

The average weight of nitrogen/100 g of streptokinase was 14.8 g. Six determinations were made and the values ranged from 14.6 to 14.9 g per 100 g of protein. The average hexose and hexosamine content was 0.20

[2] The p$K$ of the N-terminal amino acids of the SK-plasminogen reaction system was estimated to be 7.60 by determination of total uptake of base by this reaction system at a pH range of 6–9 at 0.2 intervals in the manner described by Mihalyi and Godfrey (1963).

[3] Eighty and ninety per cent of the total protein adsorbed onto the DEAE Sephadex and Sephadex G-100 columns, respectively, was recovered from the eluates.

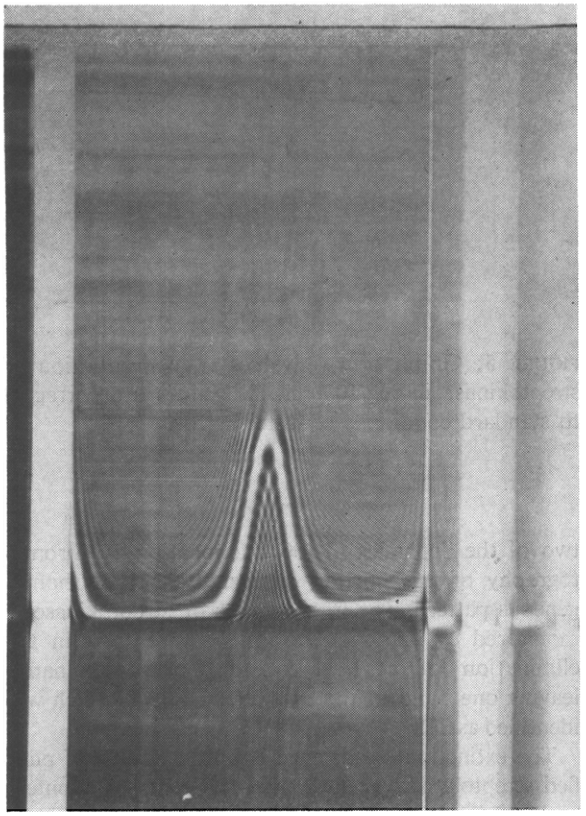

FIGURE 6: Schlieren pattern of streptokinase, 0.33% in 0.001 M phosphate buffer–0.10 M NaCl, pH 7.4, 60 min after reaching 59,780 rpm; bar angle 65°, temperature 20°, $s_{20,w} = 3.12 \times 10^{-13}$ S.

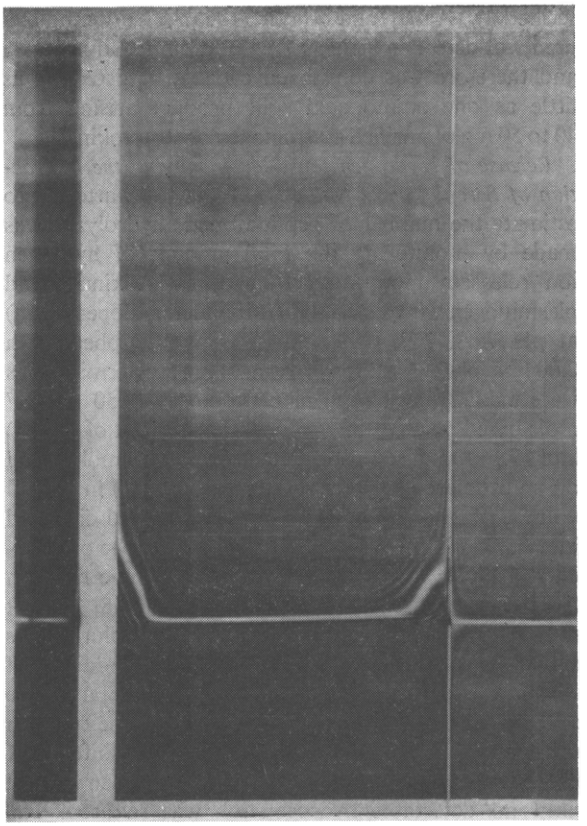

FIGURE 7: Schlieren pattern of streptokinase, 0.33% in 0.001 M phosphate buffer–0.10 M NaCl, pH 7.4, 120 min after reaching 11,720 rpm; bar angle 65°; temperature 20°.

(0.1–0.3) and 0.10 g (0.01–0.15) per 100 g of streptokinase, respectively.

Figure 8 shows the results of the interaction of 50 mg of plasminogen with 0.5 mg of streptokinase in which a polypeptide split product is produced (observed in eight out of ten runs). It was also noted in the same eight of ten runs that the plasmin formed was excluded from the Sephadex gel to a greater extent than the plasminogen control. This unusual behavior of plasmin was always associated with the appearance of a split product. Conversely in the two studies in which no split product was observed, there was no difference in the volume required to elute either plasminogen or plasmin from Sephadex G-100. The estimated molecular weight of the split product using the gel filtration technique is 6200 (Figure 4). Figure 9 shows that EACA inhibits the formation of this split product. Figure 10 shows that no split products, discernible by either spectrophotometry or ninhydrin assay, of G-100 Sephadex column eluates were produced when 50 mg of SK reacts with 0.4 mg of plasminogen. However, 1–2% by weight of the streptokinase starting material does appear after 20–30 min as trichloroacetic acid soluble material.

Titration of the total amount of hydrogen ion produced by $2.8 \times 10^{-7}$ mole of SK reacting with $7.5 \times 10^{-7}$ mole of plasminogen with the pH-Stat revealed that $3 \times 10^{-7}$ mole of hydrogen ion is produced. Based on an estimate that the p$K$ of the liberated amino acid group was 7.58, the pH of the reaction system was set at 7.58. Studies of this reaction (Figure 8) were also done at pH 9.0. From the following equation

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{NH_2\ protein}} - \log \frac{(\mathrm{NH_{3\ protein}})}{3 \times 10^{-7}}$$

the total amount of hydrogen ion liberated was calculated to be $6 \times 10^{-7}$ mole (*i.e.*, $3 \times 10^{-7}$ mole of $NH_3$ calculated to be produced plus $3 \times 10^{-7}$ mole of $H^+$ actually titrated = $6 \times 10^{-7}$ mole of total). Assuming that for every peptide bond hydrolyzed there is one hydrogen ion released, $6 \times 10^{-7}$ mole of hydrogen ion would be equivalent to $6 \times 10^{-7}$ mole of $H^+$ liberated. This approximated the total number of moles of plasminogen ($7.5 \times 10^{-7}$) present in the reaction system. This would then be equivalent to approximately one to two amino groups liberated or one to two peptide bonds hydrolyzed per molecule of plasminogen, given a range of error of $\pm 1.5 \times 10^{-7}$ mole of hydrogen ion.

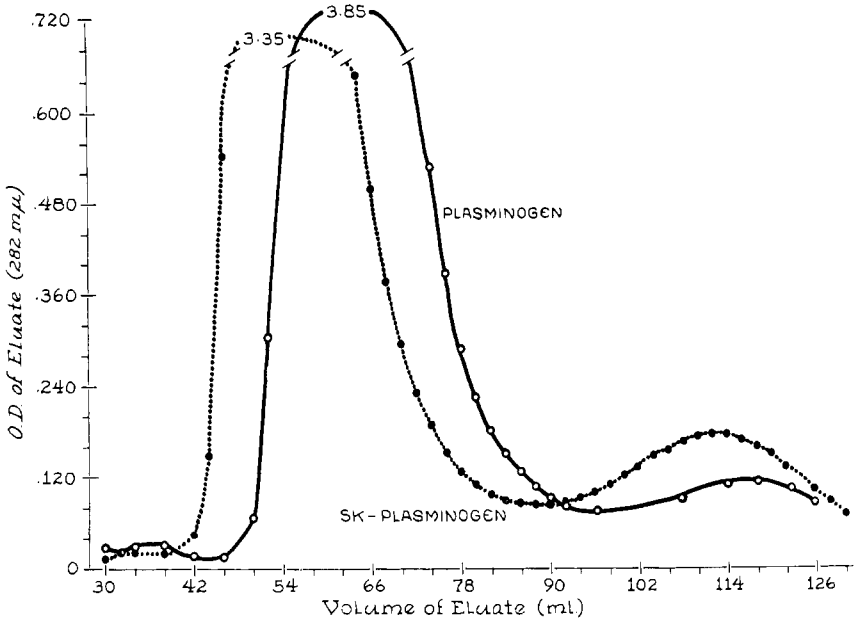


FIGURE 8: Elution patterns of 50 mg of plasminogen, 50 mg of plasminogen, and 0.4 mg of streptokinase on Sephadex G-100 in 0.001 M phosphate–0.5 M EACA–0.3 M NaCl buffer solution at pH 7.4, 4°.

## Discussion

The streptokinase starting material (Varidase) used in these studies was that which was originally extracted from β-hemolytic *Streptococcus* group C, lot 108–478 obtained from Lederle Laboratories. It should be emphasized that the electrophoretic characteristics of streptokinase extracted from other strains of hemolytic streptococci differ from that extracted from group C as has been shown by Dillon and Wannemaker (1965). It is to be expected, therefore, that the physical chemical and chemical characteristics reported herein apply only to streptokinase from group C *Streptococcus* and possibly only to lot 108–478, as batches may differ because of variation in the properties of the bacterial cultures of a given strain.

The procedure used in purification of streptokinase from Varidase has been outlined in detail because until recently (De Renzo *et al.*, 1967) little has been written on the preparation of high-purity streptokinase.[4] Dillon and Wannemaker (1965) reported that streptokinase could be prepared in relatively pure form on the basis of immunoelectrophoretic analysis by chromatography of Varidase on DEAE-cellulose. However, we found that it was necessary to employ both Sephadex G-100, to remove an $\alpha_1$-macroglubulin contaminant, and an ion-exchange cellulose with a higher charge density (DEAE Sephadex A-50, 3.5 mequiv/g), to remove a slow $\alpha_2$-globulin contaminant along with the other more negatively charged contaminants. This fractionation procedure was monitored not only by immunoelectrophoresis but also by an activity assay employing purified plasminogen and a synthetic substrate (LMe). This particular assay which we have utilized may represent a useful alternative to the

TABLE I: Amino Acid Composition of Streptokinase[a] in Moles/50,000 g (1 g mol wt) of Streptokinase.

| | |
|---|---|
| Aspartic acid | 67 |
| Threonine | 27 |
| Serine | 22 |
| Proline | 19 |
| Glutamic acid | 51.6 |
| Glycine | 24.7 |
| Alanine | 24 |
| Valine | 26 |
| Methionine | 0.6 |
| Isoleucine | 22.9 |
| Leucine | 32.6 |
| Tyrosine | 8.2 |
| Phenylalanine | 12.1 |
| Lysine | 30.2 |
| Histidine | 6.4 |
| Arginine | 18 |

[a] Digestion period was 22 hr at 110 ± 0.5°.

[4] De Renzo *et al.* (1967) (Lederle Co.) has very recently described a method different from ours of purification of SK developed for Lederle under U. S. Patent 3,226,304 (*J. Biol. Chem. 242*, 533 (1967)). Earlier, noteworthy, less extensive studies include those of Fletcher and Johnson (1957) and Blatt *et al.* (1966).

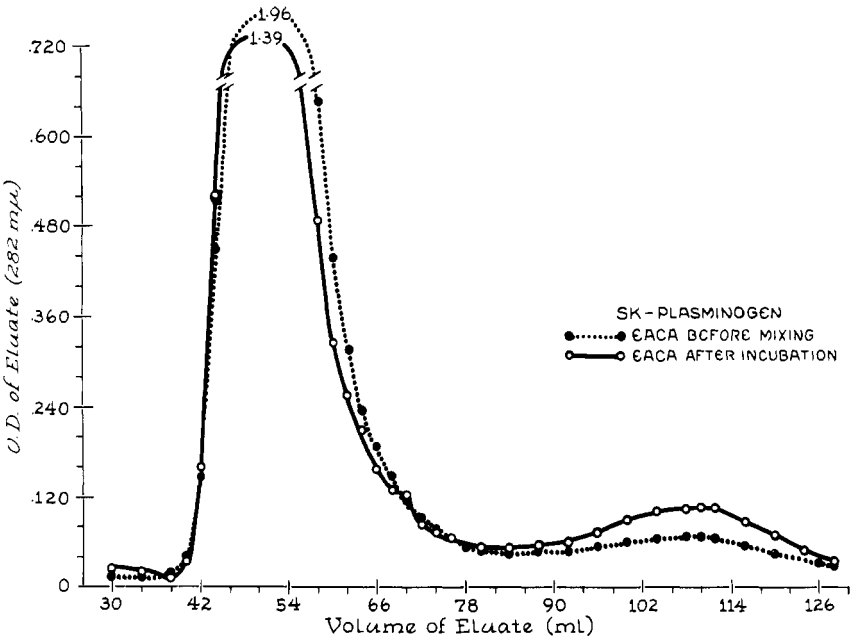


FIGURE 9: Elution pattern of 21 mg of plasminogen and 0.4 mg of streptokinase with 0.3 M EACA added *before* mixing and with 0.3 M EACA added 10 min *after* mixing on Sephadex G-100 at pH 7.4, 4°.

Christensen assay and other similar assays because (1) the end point is not based on visualization of lysis of a clot which is open to subjective error; (2) the intermediate points, other than the end point, can be determined; and (3) the standardization of the active components (plasminogen, LMe) can be controlled more closely, whereas systems employing multiple, less stable and less pure components (euglobulin or whole clot assay systems), although simpler to set up, are more difficult to standardize and compare with data from other laboratories (Johnson and Skoza, 1966).

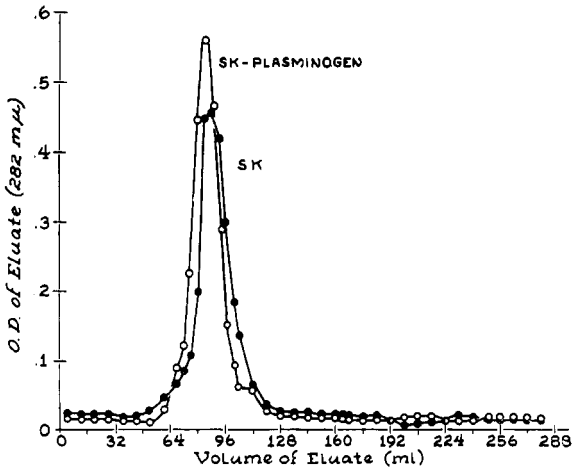


FIGURE 10: Elution pattern of 10 mg of streptokinase and 10 mg of streptokinase with 0.4 mg of plasminogen on Sephadex G-100 in 0.001 M phosphate–0.1 M NaCl buffer solution at pH 7.4, 4°.

The physical chemical data on streptokinase purified from lot 108–478 compare favorably with those reported by Davis *et al.* (1964) (*i.e.*, extinction coefficient, density, partial specific volume, viscosity, sedimentation, and velocity).[4] The values calculated from the depth of the trough of the Cotton effect suggest that a small portion (12%) of the streptokinase molecule may exist in the form of a helix. The sedimentation equilibrium and gel filtration estimates of the molecular weight of streptokinase are 50,000 ± 3000 and 55,000 ± 4000. These values are similar to the value of 47,000 obtained by Davis *et al.* (1964) as determined by sedimentation equilibrium alone. The accuracy of these two procedures in our hands is supported by the fact that the estimates of the molecular weights of ribonuclease and sucrose using these two techniques were within 10% of the values obtained by Schachman (1957) and Andrews (1964).

The observation that streptokinase contains only 0.20% hexose and 0.10% hexosamine suggests that its carbohydrate content is very low. The high proportion of glutamic and aspartic acid relative to lysine as revealed by amino acid analysis is consistent with the observation that streptokinase migrates in the $\alpha_1$-globulin region with an approximate p*I* of 4.8. Virtually no sulfur- or sulfhydryl-containing amino acids (methionine and cysteine) were detected. The above data are in general agreement with those figures recently published by De Renzo *et al.* (1967).

The reaction of purified streptokinase as described above with an excess of plasminogen yielded, in 8 out

of 10 runs, at least one split product (polypeptide of 6200 mol wt). The presence of other polypeptides of molecular weight below 2000 or above 10,000 was excluded by (1) analysis of the eluate from Sephadex G-100 beyond that which contained the polypeptide for amino acids by the ninhydrin method described previously, and (2) by rechromatography of the polypeptide through Sephadex G-25 and G-50. In neither case were amino acids (10 $\mu$g or more from 65 mg of plasminogen) or polypeptides of intermediate size detected. We therefore concluded that the principal and possibly only split product was the 6200 mol wt fragment. Shulman also studied the streptokinase–plasminogen reaction from which he isolated an acid-soluble dialyzable protein termed plasminopeptide which constituted 30% of the total protein and presumably was a split product from plasminogen conversion to plasmin (Shulman and Rive, 1964). Employing this same technique but using 0.5 mg of highly purified streptokinase to 60 mg of purified plasminogen we also detected an acid-soluble dialyzable peptide which represented, however, only *14.8%* of the initial protein. This per cent by weight of split product to starting material matches closely with that obtained in the chromatographic studies (15.0%) described above. Thus, while these results are in agreement with Shulman's qualitative findings, the per cent by weight figure of the split product relative to the starting material varied twofold from his. This may be explained in part by the fact that large amounts of relatively impure streptokinase were used in his studies, for we have found that the proteins contaminating streptokinase are hydrolyzed to small polypeptides which are also acid soluble and dialyzable. The fact that EACA inhibited the formation of this split product further suggested that its formation was associated directly with the activation of plasminogen by SK because of the demonstrated capacity of 0.15 M EACA to specifically and completely inhibit the activation step (Alkjaersig *et al.*, 1959). Also the fact that this split product appears within 5 min of mixing the reactants and does not increase in amount over the succeeding 20 min and the fact that the specific activity of plasmin does not decrease over this 20-min period further suggest that the formation of this split product is associated with some phase of SK activation of pseudoglobulin plasminogen and not with autodigestion of the plasmin formed. However, plasminogen alone (Figure 7) even after purification and chromatography on Sephadex G-100 always yields some material in a lower molecular weight range upon subsequent rechromatography on Sephadex G-100. It is assumed that this material arises from the autodigestion process (plasmin acting on plasmin), for it has been demonstrated that EACA in high concentrations (1.0 M) or 50% glyceral inhibits the "spontaneous" formation of these fragments.

These results on activation of pseudoglobulin plasminogen differ qualitatively from those described by Robbins *et al.* (1967) in which he observed that plasminogen activated by *urokinase* yielded no split products. The question of whether these differences are due to differences in materials (pseudoglobulin plasminogen *vs.* euglobulin plasminogen) or procedure (activation in glycerol *vs.* activation in phosphate–saline solution) remains to be studied, particularly in view of the fact that in two of the ten studies no split product or conformational change was observed. It may also be possible that we are observing the second event associated with the activation process whereas Robbins is observing the first event of a two-step series of reactions associated with the activation and conformational change of plasminogen. It is clear, however, from the interval studies that the 6200 mol wt fragment which we have observed is not associated with plasmin autodigestion but rather with some phase of activation of pseudoglobulin plasminogen. The only alternative is release of this fragment from a protein contaminating the plasminogen which is not detectable by immunoelectrophoresis, ultracentrifugation, or acrylamide gel electrophoresis at pH 4.5 and 8.9. In this case the contaminant would have to comprise at least 20% of the protein. This seems unlikely.

In addition to the appearance of a split product we have noted that the product plasmin is more completely excluded than plasminogen from the Sephadex gel as is shown in Figure 8. This may reflect a marked change in conformation associated with the activation step. The supposition that plasmin can undergo changes in shape is supported in part by Robbins *et al.* (1965, 1967) in which a high sedimentation value was reported for plasmin relative to plasminogen (4.8 S for plasmin *vs.* 4.4 S for plasminogen in pH 3.0, 0.001 N HCl–0.1 M NaCl solvent). Further support that the plasmin at pH 9.0 which was excluded by G-100 has undergone a change in conformation is provided by our preliminary observations of the optical rotatory dispersion patterns of plasmin and plasminogen in which plasmin has undergone an increase in the negative angle of rotation relative to plasminogen.

The reaction of plasminogen (or plasmin) with an excess of streptokinase was of particular interest because of the absence of any split products detectable by the methods described which could be attributable to hydrolysis of streptokinase. The amounts of SK employed (10–40 mg) and the sensitivity of the ninhydrin assay of the eluate digests were such that one $\alpha$-amino acid molecule liberated per molecule of streptokinase would have been detected, whereas the amino acids liberated from the small amounts of plasminogen used would not have been detected. The observation that no split products separable by gel filtration arise from SK during the initial 5-min activation period does not eliminate the possibility of a chemical event (short of release of a fragment) occurring which would alter the structure or conformation of SK. Our observations also do not eliminate the possibility that larger amounts of plasmin relative to SK acting over an extended period of time might hydrolyze SK, producing split products. In fact the recent observations of De Renzo *et al.* (1967) suggest that this is the case. This raises the interesting question of whether or not SK hydrolyzed by plasmin is also capable of activating

plasminogen. However, these studies do suggest that SK under these conditions is not readily susceptible to hydrolysis by plasmin particularly during the (3–5 min) period in which plasminogen is converted to plasmin (as determined by the pH-Stat and LMe assay). Our observations suggest further that hydrolysis of SK with a release of peptides is not associated with the activation step although we have no evidence as to whether SK modified by larger amounts of plasmin can also serve as an activator. This interpretation is supported by our data on the molar quantities of hydrogen ion released during the SK–plasminogen reaction and by the observations and conclusions of De Renzo *et al.* (1967).

The pH-Stat analysis of liberated hydrogen ion together with the above information suggest that only one and no more than two peptide bonds per molecule of plasminogen are split producing a fragment with a mol wt of 6200 and that the streptokinase itself is not hydrolyzed. From these data one might speculate that streptokinase, in forming a complex with plasminogen and/or plasmin, acts passively to redirect the specificity of the small amount of plasmin always present in plasminogen such that the plasmin–SK complex serves as activator of plasminogen capable of splitting one peptide bond per molecule of plasminogen to yield more plasmin. This concept tends to be supported by the fact that: (1) the split product arising from either plasmin (slow autocatalytic) hydrolysis of plasminogen- or SK-induced (rapid) hydrolysis are identical, (2) complex formation of SK with plasmin to form an activator of plasminogen has been demonstrated repeatedly (Davis *et al.*, 1964), (3) this complex can be recovered by lowering the pH to 3.0 (Kline and Fishman, 1961), (4) both plasmin and activator activities are inhibited by diisopropylfluorophosphate and EACA (Taylor and Staprans, 1966), and (5) the fact that no synthetic or protein substrates susceptible to SK (other than plasminogen) have been found (Buck and De Renzo, 1964). However, the possibility that SK is indeed an enzyme cannot be conclusively eliminated until the nature of the binding and chemical groupings involved in the SK–plasmin interaction are described.

## Acknowledgments

We wish to acknowledge the technical assistance of Mrs. Ilona Staprans and Mrs. Gloria Heym. The amino acid analyses were performed by Dr. John Rockey of the Department of Microbiology, University of Pennsylvania.

## References

Alkjaersig, N., Fletcher, A. P., and Sherry, S. (1959), *J. Biol. Chem. 234*, 832.

Andrews, P. (1964), *Biochem. J. 91*, 222.

Blatt, W. F., Segal, H., and Gray, J. L. (1966), *Thromb. Diath. Haemorrhag. 11*, 393.

Buck, F. F., and De Renzo, E. C. (1964), *Biophys. Biochim. Acta 89*, 384.

Davis, M. C., Englert, M. E., and De Renzo, E. C. (1964), *J. Biol. Chem. 239*, 2651.

De Renzo, E. C., Boggrono, E., Barg, W. F., and Bush, F. F. (1967a), *J. Biol. Chem. 242*, 2428.

De Renzo, E. C., Siiteri, P. K., Hutchings, B. L., and Bell, P. H. (1967b), *J. Biol. Chem. 242*, 533.

Dillon, H. C., and Wannemaker, L. W. (1965), *J. Exptl. Med. 121*, 351.

Dische, Z. (1955a), *Methods Biochem. Anal. 2*, 313.

Dische, Z. (1955b), *Methods Biochem. Anal. 2*, 500.

Fletcher, A. P., and Johnson, A. J. (1957), *Proc. Soc. Exptl. Biol. Med. 94*, 233.

Handbook of Chemistry and Physics (1965), Hodgman, C. D., West, C. R., and Selby, S. M., Ed., 48th ed, Cleveland, Ohio, Chemical Rubber, Publishing Co.

Jirgenson, B. (1966), *J. Biol. Chem. 241*, 147.

Johnson, A. J., and Skoza, T. (1966), *Federation Proc. 25*, 34.

Kabat, E. A., and Mayer, M. M. (1961), Experimental Immunochemistry, 2nd ed, Springfield, Ill., C. C Thomas, p 561.

Kline, D. L., and Fishman, J. B. (1961), *J. Biol. Chem. 236*, 2807.

Mihalyi, E., and Godfrey, J. E. (1963), *Biochim. Biophys. Acta 67*, 73.

Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1125.

Robbins, K. C., Summaria, L., Elevin, D., and Barlow, G. H. (1965), *J. Biol. Chem. 240*, 541.

Robbins, K. C., Summaria, L., Hsieh, B., and Shah, J. R. (1967), *J. Biol. Chem. 242*, 2333.

Roberts, P. S. (1962), *Can. J. Biochem. Physiol. 40*, 1203.

Shulman, S., and Rive, U. (1964), *Biochim. Biophys. Acta 86*, 309.

Simmons, N. S., Choen, C., Szent-Gyorgyi, A. G., Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.

Taylor, F. B., and Staprans, I. (1966), *Arch. Biochem. Biophys. 114*, 26.

Timmermans, J. (1960), The Physco-Chemical Constant of Binary Systems in Concentrated Solutions, Vol. 4, New York, N. Y., Interscience, p 495.

Willits, C. O., Cos, M. R., and Ogg, C. L. (1949), *J. Assoc. Offic. Agr. Chemists 32*, 118.