Preparation and Certain Properties of Highly Purified Streptokinase

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SUMMARY

Procedures are described for the preparation of highly purified streptokinase by column chromatography on diethyaminoethyl cellulose and by column electrophoresis in a sucrose density gradient. Preparations chromatographed at least twice on diethyaminoethyl cellulose are shown to be essentially monodisperse on the basis of ultracentrifugal and gel electrophoretic analyses and constancy of specific activity. In good agreement with a previous finding from this laboratory, the molecular weight determined by equilibrium sedimentation was found to be 47,600. Treatment of streptokinase at pH 7.5 in 0.1 M phosphate buffer with 5 M guanidine-hydrochloride or 8 M urea produced a lowering of the sedimentation coefficient without significantly changing the molecular weight. Cystine and cysteine were absent on amino acid analysis, and the molecule is therefore assumed to consist of a single polypeptide chain with no subunits. The isoelectric point was found to be about pH 4.7.

The amino acid composition is consistent with the formula Asp_80- Thr_16- Ser_14- Gln_13- Pro_13- Gly_12- Ala_12- Val_12- Met_12- Ile_12- Leu_12- Tyr_12- Phe_12- Lys_12- His_12- Arg_12- Thr_1 for molecular weight 47,754. Approximately 60 aspartic and glutamic residues are amidated per molecule of protein. The most highly purified preparations are devoid of carbohydrate and phosphorus and are inactive with basic amino acid esters, naphthyl esters, and acetytyrosine ethyl ester as substrates.

In the early 1930's Tillett and Garner (1) described an extracellular substance, streptokinase, which was elaborated during the growth of certain hemolytic streptococci and which brought about the lysis of human fibrin clots by activating a proteolytic system of human blood. Especially in the last decade there has been considerable interest in the possible use of streptokinase as human plasminogen to form plasmin appears to be unique so far as activation of a proteolytic enzyme precursor is concerned, and considerable effort has been expended in several laboratories in order to elucidate the sequence of events involved in the process of activation.1

Both for potential clinical usefulness in man and for chemical studies of its interaction with human plasminogen, it was obviously desirable to obtain highly purified preparations of streptokinase. Purification studies were therefore undertaken in our laboratory with the use of commercially available impure streptokinase preparations as starting materials. This paper presents detailed procedures for purifying streptokinase by chromatography on diethyaminoethyl cellulose and by density gradient electrophoresis. The most highly purified preparations have been shown to be essentially monodisperse on the basis of ultracentrifugal and gel electrophoretic analysis. The amino acid composition and other distinguishing properties of streptokinase are also given.

EXPERIMENTAL PROCEDURE

Materials

Organic compounds and sucrose were Merck reagent grade chemicals. Tris was the highest purity available from Sigma.

Streptokinase Preparations—The following crude preparations designated Preparations A, B, C, and D were used as starting materials.

Preparation A was obtained by exhaustively dialyzing streptokinase preparations (marketed under the tradename Varidase by Lederle Laboratories Division, American Cyanamid Company) against distilled water and freeze-drying the solution after adjusting it to pH 7.5 with 1 N NaOH.

Preparation B was a 16% ethanol precipitate of Varidase obtained by dissolving Preparation A in a minimum volume of water at pH 7.5 to 8.0, adding sodium acetate to 0.04 M concentration, adjusting the pH to 5.2 with glacial acetic acid, and adding ethanol to 16% concentration (v/v) at -4°. The precipitate was collected by centrifugation and suspended in a minimum volume of water. The suspension was brought to pH 5.5 to 6.0 with 1 N NaOH, and after stirring for 1 hour, any

1 For a review on plasminogen and its activation to plasmin by streptokinase, the interested reader is referred to an article by Ablondi and Hagan published in 1961 (2). The more current literature dealing with this subject can be obtained from several papers published in the last few years (3-7) and in parts of two review articles (8, 9). A general discussion of fibrinolysis and the importance of the plasminogen-plasmin system in health and disease is given in a comprehensive article by Sherry, Fletcher, and Alkjaersig (10).
The filtrate was adjusted to pH 7.0 to 7.5 by the addition of 1 N HCl, dialyzed exhaustively against distilled water, red-juted to pH 7.5 to 8.0, and finally lyophilized.

Preparation C preparations were derived from Preparations A and B above by a batch adsorption-elution process on DEAE-cellulose in the following way. Preparations A or B were dissolved at pH 7.0 to 7.5 in distilled water (protein concentration 10 to 50 mg per ml) and DEAE-cellulose, conditioned with 0.01 to 0.04 M phosphate buffer, pH 7.0, was added. (Approximately the equivalent of 1 g of dry DEAE-cellulose was added per 200 mg of protein.) Adsorption was allowed to take place while the suspension was stirred for 50 min at 20°. The DEAE-cellulose was filtered off, washed on the funnel with a few small portions of acetone-carbonate mixture, and discarded. When the pH remained constant for 15 min, the cellulose was filtered and stirred in phosphate buffer of the desired concentration for 30 min and then filtered. The elution step was repeated two to three times, and the conditioned DEAE-cellulose was then suspended in the same buffer and packed into the column as described by Sober et al. (12).

Methods

Column Chromatography—Gradient and stepwise elutions were carried out under a variety of conditions. The details of each experiment are given with the experimental results. In all cases the sample applied to the column was pre-equilibrated with buffer used to condition the DEAE-cellulose by dialysis against several changes of the buffer for 24 to 48 hours. All manipulations were carried out at 4° and effluent fractions were collected with the aid of a Technicon fraction collector. Flow of the buffer through the columns was adjusted with the aid of an air pressure line. Flow rates were generally about 4 to 8 ml per cm² per hour.

Density Gradient Electrophoresis—The column employed was designed in our laboratory and is shown schematically in Fig. 1. Linear sucrose density gradients were formed with the aid of a scaled up version of the mixing device described by Britten and Roberts (13) for establishing gradients in centrifuge tubes. A layer of 50% sucrose in buffer was first placed in the bottom of the column, and the outflow vessel of the mixing device contained the same solution. The second mixing vessel contained buffer without sucrose. The gradient was built to a height of 40 to 70 cm. To provide for introduction of the sample, the flow of gradient into the column was interrupted at a point about 80% of the desired column height, the next 2 ml of the gradient were diverted into a test tube, and the flow was stopped. The 2-ml aliquot was diluted with an equal volume of sucrose-free buffer and 2 to 3 ml of the mixture were used to dissolve the sample. The liquid remaining in the gradient mixing vessels was then diluted by adding an equal volume of buffer to the vessel which originally contained buffer alone and allowing the contents of both vessels to intermix under the influence of gravity and attain a new equilibrium level. The building of the column was then continued with this new gradient mixture. The sharp discontinuity thus formed in the gradient column was easily visible and the sample was inserted at this point with the aid of a hypodermic syringe and polyethylene capillary tubing. As rapidly as possible thereafter, buffer was layered over the top of the column and into the side arm. The column was closed off at the top, and the anode vessel was placed at the bottom of the column. The liquid level in the leveling bulb was adjusted to match that of the anode vessel and electrophoresis was started at once. All experiments were performed in a 4° cold room with -1° alcohol-water circulating in the column-cooling jacket. At
the conclusion of the experiment the current was shut off, the anode vessel was removed, the bottom membrane was punctured with a needle, and samples were collected dropwise, either manually or with a drop-counting fraction collector. The rate of emptying of the column contents could be controlled very critically by adjusting the height of the leveling bulb attached to the column. Generally, 5-ml fractions were collected.

**Protein Measurement**—For the determination of protein concentrations, the absorbance of solutions was measured at 280 \( \text{nm} \) in a Beckman-DU spectrophotometer through a 1-cm light path. For conversion to milligrams of protein per ml, an \( E_{1\%}^{1\text{cm}} \) value of 10 was assumed. For the purer preparations (C and D) and for highly purified streptokinase, the assumed extinction coefficient gives a fairly accurate measure of protein concentration.

**Calculation of Specific Activity**—Appropriate fractions or their pools were checked for streptokinase activity as described by Christensen (14). Specific activity was calculated as units of activity per mg of protein or as units per \( \mu \text{g} \) of nitrogen. The specific activities of the starting materials were as follows: Preparation A, 5 to 8 \( \times 10^3 \) units per mg of protein (50 to 80 units per \( \mu \text{g} \) of nitrogen); Preparation B, 8 to 10 \( \times 10^3 \) units per mg of protein (90 to 110 units per \( \mu \text{g} \) of nitrogen); Preparation C, 15 to 18 \( \times 10^3 \) units per mg of protein (90 to 110 units per \( \mu \text{g} \) of nitrogen); and Preparation D, 25 to 40 \( \times 10^3 \) units per mg of protein (150 to 240 units per \( \mu \text{g} \) of nitrogen). These analyses suggested that the most crude preparations (A and B) were contaminated with ultraviolet-absorbing materials (probably nucleotides or nucleic acids, see later).

**Starch Gel Electrophoresis**—The procedure followed has been described in previous communications from this laboratory (7, 15) except that the buffer composition of the gel and the electrode baths was 0.1 M Tris-0.05 M boric acid, pH 7.5. Appropriate effluent samples were prepared for electrophoresis by first dialyzing them exhaustively against distilled water, readjusting the pH to about 7.5 with dilute alkali, and then lyophilizing. The powders were dissolved in the Tris-boric acid buffer at a concentration of about 10 mg per ml.

**Velocity Sedimentation and Equilibrium Sedimentation Experiments**—These were performed and analyzed as described previously (6). In all cases samples were equilibrated by dialysis against the desired buffer before sedimentation. In experiments in 5 M guanidine-hydrochloride or 8 M urea, 0.1 M phosphate was also present and final pH adjustment was made to 7 to 7.1 with 1 N NaOH.

**Determination of Isoelectric Point**—Electrophoresis was carried out in a Spinco model II apparatus at 5°, and mobilities were calculated from enlarged projections of the schlieren patterns. The pH range studied was limited because of the marked insolubility of streptokinase near its isoelectric point (between pH 4.0 and 6.0). The following buffers were used: 0.05 M glycine-0.05 M NaCl-0.012 M HCl at pH 3.0; 0.01 M glycine-0.1 M NaCl-0.0005 M HCl at pH 3.5; 0.02 M sodium cacodylate-0.01 M cacodylic acid-0.08 M NaCl at pH 6.28; and 0.008 M NaH$_2$PO$_4$-0.0037 M Na$_2$HPO$_4$ at pH 7.66. In all cases the sample was dissolved and equilibrated with the desired buffer by exhaustive dialysis against the appropriate buffer.

**Amino Acid Analyses**—Quantitative amino acid analyses were performed on acid hydrolysates of the most highly purified preparations of streptokinase which were subjected to column chromatography on DEAE-cellulose at least three times. Even then, only the central portion of the streptokinase peak was taken for analysis. The samples were dialyzed exhaustively against distilled water and readjusted to pH 7 to 7.5 prior to lyophilization. A Technicon amino acid analyzer was used for the amino acid analysis and the procedure followed was that described by Boyer and Talalay (16). The procedure for preparation of the hydrolysates was that of Crestfield, Moore, and Stein (17). Tryptophan was determined spectrophotometrically (18) and colorimetrically (19) in the intact protein.

**Calculation of Amino Acid Composition**—The quantities of each amino acid were calculated by graphically integrating the area under the peaks. In numerous analyses of a standard amino acid mixture (Technicon), the areas of each amino acid (0.25 \( \mu \text{mole} \) of each) were compared with that obtained with 0.25 \( \mu \text{mole} \) of norleucine, which was added to each sample analyzed and served as an internal control. The mean color coefficient ratios (area of 0.25 \( \mu \text{mole} \) of each amino acid divided by the area of 0.25 \( \mu \text{mole} \) of norleucine) were as follows: Asp, 0.951; Thr, 0.975; Ser, 0.998; Glu, 0.986; Pro (at 440 \( \text{mu} \)) 0.272; Gly, 1.12; Ala, 0.915; Val, 0.886; Met, 0.956; Ile, 0.916; Leu, 1.05; Tyr, 1.03; Phe, 0.984; Lys, 1.17; His, 1.10; and Arg, 1.01. Several of these values (those for the Thr, Ala, Val, and Ile) significantly differ from those reported by Boyer and Talalay (16).

Since identical amounts of protein were not taken for analysis for each time of hydrolysis, losses occurring during hydrolysis were determined by plotting millimicromoles of amino acid recovered per \( \mu \text{g} \) of nitrogen applied to the column from hydrolysates of streptokinase obtained after 23, 48, 72, and 96 hours.
Molar ratios were calculated in relation to valine or histidine after normalization of the data (see later under "Results"). With each amino acid, the calculated relative molar ratios were multiplied by the whole number \( R \) which yielded a number of residues for each amino acid, the sum total of the weight of which most closely approached the experimentally determined value of the molecular weight 47,000 (6). \( R \) values for valine and histidine were 23 and 29, respectively.

**Chemical and Enzymatic Tests**—Carbohydrate (21) and phosphorus content (22) were determined after exhaustive dialysis of the most highly purified preparations against 0.001 m HCl. Streptokinase activity remains intact after the acid treatment. Lysine-methyl esterase (23), tosyl-arginine methyl esterase (24), acetyl-tyrosine ethyl esterase (25), and naphthyl acetate esterase (26) activities were performed on various preparations as desired.

**RESULTS**

**Purification of Streptokinase by Column Chromatography on DEAE-cellulose**—Fig. 2 shows a typical elution pattern obtained with a streptokinase Preparation B subjected to linear gradient elution between the limits of 0.05 m phosphate at pH 6.0, and 0.1 m NaH2PO4-0.2 m NaCl. (In Fig. 2 and ensuing figures the streptokinase activity is shown only for the main peak. In fact, detectable but minor amounts were found in practically all effluent fractions.) In this experiment the streptokinase yield was essentially quantitative and an over-all purification of about 5-fold was achieved. Fig. 3 shows the results of another column with the same streptokinase Preparation B wherein a decreasing pH and increasing ionic strength gradient were employed for elution. In this experiment, the limit buffer was 0.1 m NaH2PO4-0.2 m NaCl. As in the previous case, good resolution of streptokinase was achieved with evidence for the separation of several other components. Over-all recovery of streptokinase was about 75%, and a 6-fold increase in purity was achieved.

The results of rechromatography of the streptokinase-rich fractions shown in Figs. 2 and 3 are given in Figs. 4 and 5.
Additional separation of impurities was achieved in both cases. It has been a consistent observation of these studies that after chromatography of streptokinase preparations twice on DEAE-cellulose under a variety of conditions the specific activity of the streptokinase-rich fraction approached a value of 90,000 to 100,000 units per mg of protein or 500 to 600 units per µg of nitrogen. Another reproducible finding was the fact that repetitive chromatography was necessary in order to remove the last detectable trace of impurities, which suggests that very closely related or highly associating proteins are present in the crude streptokinase preparations. Upon rechromatographing for the third time, essentially a single peak was obtained with only minor impurity peaks on the fore and back side of the main peak.

Streptokinase Preparations C and D have also been purified by the procedures described above. As expected with such preparations, the elution patterns were less complicated in the impurity areas than those shown in Figs. 2 and 3. However, final degree of purification and over-all yield was essentially the same as that shown in Figs. 2 to 5.

An example of purification achieved by stepwise elution is given in Fig. 6. In this experiment streptokinase Preparation D was used and the diagram shows that elution of the streptokinase was accomplished with 0.075 M phosphate, pH 8.0, following the removal of impurities at lower ionic strength. Some variability in the results under these conditions has been observed which appeared to be primarily dependent upon the batch of DEAE-cellulose employed. Thus with certain commercial batches of DEAE-cellulose, streptokinase began to emerge from the column immediately following the impurity area with the use of 0.06 M phosphate. More generally, however, the streptokinase peak tailed even more than shown in Fig. 6, and it was convenient to elute the streptokinase with 0.1 M phosphate, pH 7.0 to 8.0, in place of the 0.075 M buffer so as to sharpen up the peak and obtain the streptokinase in more concentrated solution. No major contamination with more tightly binding impurities was brought about by such a treatment. As in the case of the gradient elution examples shown in Figs. 2 to 5, repetitive chromatography removed additional impurities.

In order to obtain streptokinase in the most highly purified form, the impure preparations were chromatographed two or three times. Generally, the experimental conditions were altered from column to column in an attempt to take maximum advantage of resolving power due to the particular conditions of ionic strength and pH. One such sequence, for example, involved successively chromatographing under the conditions shown in Figs. 1, 2, and 6. Such thrice chromatographed streptokinase preparations consistently displayed a specific activity of approximately 100,000 units per mg of protein or about 600 to 700 units per µg of nitrogen. Table I gives a summary of other experimental conditions used to purify streptokinase by chromatography on DEAE-cellulose.

<table>
<thead>
<tr>
<th>Column conditioning buffer</th>
<th>Elution conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04 M phosphate, pH 7.0</td>
<td>Stepwise elution with 0.06 M phosphate, pH 7.5, followed by 0.1 M phosphate, pH 7.0</td>
</tr>
<tr>
<td>0.04 M phosphate, pH 7.0</td>
<td>0.075 M phosphate, pH 8.0, followed by gradient elution between the limits of 0.095 M phosphate, pH 6.8, and 0.2 M NaH₂PO₄, 0.2 M NaCl</td>
</tr>
<tr>
<td>0.095 M phosphate, pH 6.8</td>
<td>Gradient elution between the limits of 0.095 M phosphate, pH 6.8, and 0.2 M NaH₂PO₄, 0.2 M NaCl</td>
</tr>
<tr>
<td>0.095 M phosphate, pH 6.8</td>
<td>0.095 M phosphate, pH 6.8</td>
</tr>
</tbody>
</table>

Despite the variability in the protein elution profile, suitable monitoring of the effluent fraction for streptokinase activity permits the proper selection of the streptokinase-rich fractions.
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Fig. 7. Diagram showing the ultraviolet absorbance of fractions obtained after electrophoresis in a sucrose density column without a protein charge. Electrophoresis was carried out for 21.5 hours at 11 volts per cm. Column size was 2.5 × 30 cm; buffer, 0.03 M borate at pH 8.7; sample charge, 1 ml of buffer; and fraction volume, 5 ml.

Fig. 8. Diagram showing the electrophoresis of a streptokinase Preparation A (see "Experimental Procedure") in a sucrose density gradient. — — absorbance at 280 nm; --- streptokinase activity. Electrophoresis was carried out for 17.1 hours at 12.5 volts per cm. Column size was 2.5 × 62 cm; buffer, 0.013 M Tris-0.16 M boric acid at pH 7.2; sample charge, 73 mg (specific activity, 12,000 units per mg of protein) in 2 ml of buffer; and fraction volume, 5 ml. The pool of Fractions 40 to 42 had a specific activity of 61,000 units per mg of protein and represented 50% of the charged streptokinase.

Sucrose Density Gradient—Figs. 8 to 10 show the results of typical experiments in which streptokinase preparations of varying degrees of purity were subjected to electrophoresis in a sucrose density gradient column. Fig. 7 is the effluent pattern of a column which was charged with 1 ml of buffer. It was necessary to perform this control experiment in order to correctly interpret the subsequent experiments since considerable ultraviolet-absorbing material (presumably present as a contaminant, or contaminants, in the sucrose) can be seen to migrate toward the anode.

Figs. 8, 9, and 10 show the results of experiments with streptokinase preparations of varying degrees of purity. As may be seen, streptokinase Preparations A and D were considerably purified, the former about 5-fold, the latter about 2-fold, and in both cases a high yield of streptokinase was obtained. Electrophoresis of streptokinase Preparations D consistently yielded a purified product in the main streptokinase peak which was similar to that obtained by purification of such preparations on a single column of DEAE-cellulose. Fig. 10 shows that electrophoresis of a preparation which was previously chromatographed on a column of DEAE-cellulose did not produce an increase in specific activity. However, even in this case, appropriate pooling of the fractions and analysis by starch gel electrophoresis demonstrated that the streptokinase-rich fraction was freed of some of the minor components present in the product chromatographed once.

One way of obtaining highly purified product was to subject an impure preparation to electrophoresis in a sucrose density column first and then fractionate the streptokinase-rich fraction further on DEAE-cellulose by one of the procedures given in Figs. 2 to 6 or Table I. Streptokinase is easily recovered from the sucrose, Tris-boric acid mixture used in the column electrophoresis by adding DEAE-cellulose (previously conditioned to 0.04 M phosphate, pH 7.0) to the streptokinase-rich pool and stirring for 1 hour, under which conditions the streptokinase is quantitatively adsorbed. Tris-boric acid, and sucrose are completely removed by filtration of the DEAE-cellulose and resuspending the adsorbed streptokinase...
advantage of exploiting two properties of proteins, namely, net charge and local charge distribution (inter alia) for purposes of purification. In fact, however, it was not possible to distinguish between a highly purified product prepared by repetitive column chromatography on DEAE-cellulose or by a sequence of density gradient electrophoresis and chromatography on DEAE-cellulose.

Homogeneity and Molecular Weight of Highly Purified Streptokinase—Ultracentrifuge patterns of streptokinase preparations chromatographed two or three times on DEAE-cellulose have been previously described from this laboratory (6). A single sedimenting boundary with $s_{20,w}$ of 3.2 S was observed in phosphate buffer, pH 7.5, over a wide concentration range, and a molecular weight of 47,000 was calculated from sedimentation and diffusion data. A value of 0.75, experimentally determined by osmometry, was used for the partial specific volume in the calculation of the molecular weight.

In the present work the molecular weight was checked by the method of Yphantis (27) which yielded a value of 47,000. Further, the molecular weight was unchanged in the presence of 5 M guanidine hydrochloride or 8 M urea at pH 7.0. In the former case the molecular weight was found to be 50,300, and in the latter it was 47,600. In both cases the sedimentation coefficient was significantly lowered (1.68 S in guanidine hydrochloride and 1.8 S in urea after correcting for the viscosity and density of the buffer). Presumably extensive unfolding of the molecule occurs in the presence of the denaturing agents without causing a reduction in the molecular weight. It is of some interest that full streptokinase activity is recovered upon dilution of streptokinase solutions maintained in 5 M guanidine hydrochloride or 8 M urea at neutral pH for 24 to 48 hours at 20$^\circ$.

Starch gel electrophoretic analysis confirmed the essentially monodisperse nature of preparations which were chromatographed at least twice on DEAE-cellulose. Electropherograms of such preparations in buffers containing 6-aminocaproic acid have been previously published from this laboratory (7), and electrophoresis in Tris-borate buffers confirmed these results. It should be stated that one or two trace components were discernible in some preparations even after repetitive chromatography on DEAE-cellulose. In general, however, there was excellent correspondence between the results of sedimentation and gel electrophoretic analyses and specific activity determinations. Preparations of streptokinase with a specific activity of about 100,000 units per mg of protein consistently were essentially monodisperse on the basis of both criteria.

Amino Acid Composition of Highly Purified Streptokinase—As shown in Table II, cystine and cysteine were not detected in the amino acid analysis. The absence of cysteine or cystine was confirmed by performing an analysis on the acid-hydrolyzed protein which was previously subjected to reduction and carboxymethylation by the procedure of Sela, White, and Anfinsen (28). Carboxymethylcysteine was not detected in this analysis.

The data of Table II show further that reasonably good reproducible results were obtained at all hydrolysis times for all the amino acids, with the exception of methionine, and for serine in phosphate buffer once or twice, filtering the suspension, and discarding the filtrates. The streptokinase may then be eluted with 0.1 M phosphate. Alternatively, if it is desired to purify the streptokinase further under conditions shown in Fig. 5, the adsorbate may be added directly to the top of a column previously conditioned with 0.04 M phosphate and, after packing it into the column, elution may be carried out.

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Amount recovered in hydrolysates at</th>
<th>Average or corrected value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
<td>48 hrs</td>
</tr>
<tr>
<td>Asp</td>
<td>8.16</td>
<td>8.09</td>
</tr>
<tr>
<td>Thr</td>
<td>3.47</td>
<td>3.26</td>
</tr>
<tr>
<td>Ser</td>
<td>2.71</td>
<td>2.34</td>
</tr>
<tr>
<td>Glu</td>
<td>5.54</td>
<td>5.66</td>
</tr>
<tr>
<td>Pro</td>
<td>2.42</td>
<td>2.53</td>
</tr>
<tr>
<td>Gly</td>
<td>2.55</td>
<td>2.50</td>
</tr>
<tr>
<td>Ala</td>
<td>2.73</td>
<td>2.65</td>
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<tr>
<td>Val</td>
<td>2.79</td>
<td>2.80</td>
</tr>
<tr>
<td>Met</td>
<td>0.28</td>
<td>0.26</td>
</tr>
<tr>
<td>Ile</td>
<td>2.66</td>
<td>2.65</td>
</tr>
<tr>
<td>Leu</td>
<td>4.98</td>
<td>4.97</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.34</td>
<td>2.43</td>
</tr>
<tr>
<td>Phe</td>
<td>1.86</td>
<td>1.84</td>
</tr>
<tr>
<td>His</td>
<td>0.40</td>
<td>0.63</td>
</tr>
<tr>
<td>His$^\alpha$</td>
<td>4.00</td>
<td>4.01</td>
</tr>
<tr>
<td>His$^\beta$</td>
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<tr>
<td>Arg</td>
<td>2.91</td>
<td>2.97</td>
</tr>
<tr>
<td>Thr</td>
<td>0.13a</td>
<td></td>
</tr>
</tbody>
</table>

* Nitrogen was determined in an aliquot of the various samples by the method of Minari and Zilversmit (20).
+ This value was corrected for decomposition during hydrolysis.
This value was determined colorimetrically (19) in the intact protein.

and threonine for which decomposition was expected. Likewise, the ammonia value was erratic. It is pertinent to note that the recovery of nitrogen in the sample analyzed for each time of hydrolysis was in excellent agreement with the amount calculated by summing the contribution of nitrogen of the individual amino acids determined in the amino acid analysis. Thus the recoveries of nitrogen in the amino acid analyses at 23, 45, 72, and 96 hours were 98.4%, 99.9%, 97.9%, and 98.1%, respectively, of the amount of nitrogen taken for analysis (30 to 50 $\mu$g).

Molar ratios were calculated from Column 5 of Table II. The use of valine as the reference amino acid are given in Table III. Approximately 1 residue of tryptophan per 47,000 molecular weight was determined colorimetrically (19), although a value closer to 2 was determined spectrophotometrically (18). In addition, in the latter analysis a value of 26 residues of tyrosine was determined per molecular weight of 47,000, and this observation, coupled with the fact that no apparent loss of tyrosine was observed during the course of hydrolysis, leads to the suspicion that the values for both these amino acids should be considered with some reservation. All facts considered, however, it is felt that the colorimetric determination for tryptophan and the amino acid analysis data for tyrosine are the more valid determinations, and hence the probable formula for streptokinase would appear to be Asp-Thr$^\alpha$-Ser$^\beta$-Glu$^\gamma$-Pro$^\delta$-Gly$^\alpha$-Ala$^\beta$-Val$^\gamma$-Met$^\alpha$-Ile$^\beta$-Leu$^\gamma$-Try$^\beta$-Phe$^\alpha$-Lys$^\beta$-Arg$^\delta$-Try$^\alpha$, for a total of 410 residues of molecular weight of 47,754. The value of 61 for amidated glutamic and aspartic residues is, of course, approximate.

The data of Table II were calculated with respect to histidine, the number of residues per molecule differed from that shown in Table III only in that 1 more glutamic acid and 1 more...
and arginine was calculated to be present per molecule for a total of 414 residues of molecular weight 47,195.

Miscellaneous Physical and Chemical Analyses of Streptokinase Preparations—The absorption spectrum of highly purified streptokinase was characteristic of a nonconjugated protein and an $A_{260}$:$A_{280}$ ratio of 2.0 was observed in 0.1 N HCl and in water at pH 7.5. Tests for carbohydrate and phosphorus were negative with the highly purified product tested at 5 to 10 mg (0.1 to 0.2 μmol). The carbon to nitrogen ratio of highly purified streptokinase was found to be 3.11:1. This agrees fairly well with the theoretical carbon to nitrogen ratio of 3.04:1 calculated from the amino acid composition given in Table III, thus suggesting that the highly purified preparations were not contaminated with major amounts of other carbon or nitrogen compounds. Certain of the impure preparations (Preparations A, B, and to a lesser extent C) showed positive tests for both carbohydrate and phosphorus in variable amounts suggesting some contamination with nucleotide or nucleic acid. This was further borne out by the lowered $A_{260}$:$A_{280}$ ratio (1.2 to 1.4) observed with such preparations. In general, one pass through a DEAE-cellulose column raised the $A_{260}$:$A_{280}$ ratio of the streptokinase peak to approximately 2.0.

The isoelectric point of purified streptokinase was found to be about 4.7. The data from which this value was derived are shown in Fig. 11. This value of 4.7 cannot be considered to be firmly established since the protein is markedly insoluble even at low concentrations (<2 mg per ml) near its isoelectric point, and mobilities could not be determined with certainty below pH 6 and above pH 3.5.

Enzymatic Analyses of Streptokinase Preparations—Crude preparations of streptokinase show considerable activity with aryl esters as substrates (26, 29, 30). Preparations D (referred to as Preparation II in the paper of Buck and De Renzo (26)) show particularly high aryl esterase activity. On the other hand, highly purified preparations were completely devoid of aryl esterase activity. The esterase activity is readily separated from streptokinase by chromatography on DEAE-cellulose. For example, Fractions 75 to 180 in Fig. 6 were high in esterase activity. The esterases have been further differentiated from streptokinase on the basis of their rapid inactivation by brief treatment with heat or with diisopropyl phosphofluoridate. Streptokinase is stable to both of these treatments (26).

Both crude and highly purified streptokinase preparations were tested for lysine and arginine esterase activities and were inactive with these substrates. Similarly no activity of highly purified streptokinase could be shown with acetyl tyrosine ethyl ester as substrate.

DISCUSSION

Although streptokinase was discovered about 35 years ago, relatively little has been published on the preparation of highly purified material. Several patents (Patents 2,997,425; 3,107,203; and 3,226,304) have been issued for purifying streptokinase with the use of DEAE-cellulose but, with one exception, Patent 3,226,304, characterization of the purified products has been meager. Fletcher and Johnson utilized alcohol fractionation, adsorption on calcium phosphate gel or Celite, and ammonium sulfate fractionation techniques to purify partially streptokinase from Varidase (31). Blatt et al. (32) obtained small amounts of purified streptokinase by subjecting Varidase to paper electrophoresis followed by chromatography on DEAE-cellulose.
According to Blatt et al., their preparation compared favorably with a highly purified material furnished by this laboratory.

The column chromatographic procedures described in Figs. 2 to 6 and Table I of the present paper have been successfully carried out with streptokinase of intermediate purities on columns (4 x 40 to 60 cm) containing 100 to 150 g of DEAE-cellulose and charged with 3 to 7 g of protein (2 x 10^9 units of streptokinase).

Experience in our laboratory with larger columns (6 to 9 x 60 cm) has been more limited, but results generally have been similar to those described for the smaller columns. Hence, methods for preparing several hundred milligram quantities of highly purified streptokinase are now available. As pointed out previously, in order to obtain the most purified product, it has been necessary to chromatograph the preparations at least twice and preferably three times in order to remove small amounts of what are presumed to be closely similar or highly associating proteins.

The results of the present paper also show that purified preparations can be achieved by density-gradient electrophoresis. Bernheimer (33) has reported further purification of highly purified streptokinase preparations by sucrose density gradient electrophoresis. The preparation that he studied was a twice chromatographed sample supplied by this laboratory, but the interpretation of his results appears to be in doubt since no correction for baseline ultraviolet absorption (see Fig. 7 of the present paper) was made in his studies. Highly purified streptokinase made by carrying out a column electrophoretic separation followed by chromatography on a DEAE-cellulose column has yielded preparations which were indistinguishable from those prepared by repetitive chromatography on DEAE-cellulose.

Preparations having a specific activity of about 100,000 streptokinase units per mg of protein have been found to be essentially monodisperse regardless of the method of preparation. They displayed one major band and one or two minor bands in gel electrophoresis and a single boundary on sedimentation analysis (6, 7). Chemical and enzymatic analyses of the most highly purified preparations have shown them to be free of carbohydrate and phosphorus and to be devoid of activity with lysine or arginine esters, naphthyl esters, and acetyl-tyrosine ethyl ester. The isoelectric point was determined to be about pH 4.7.

The molecular weight of 47,600 determined by equilibrium sedimentation is in good agreement with the value of 47,000 for the molecular weight determined by sedimentation in the present studies was derived by using an experimentally determined value of 0.75 for the partial specific volume. Based on the amino acid analyses of the present paper, a value of 0.730 for the partial specific volume was calculated (34). The reason for this discrepancy is unclear. However, in view of this variance, the formula for streptokinase given above may be in error since calculation of the molecular weight by applying the Svedberg equation and employing the values for the sedimentation coefficient and diffusion constant reported previously (6) and a partial specific volume of 0.730 would yield a molecular weight of 43,500.

In any event, the molar ratios reported in the present work would seem to be reasonably well established and should serve as a guide for any possible future changes in the actual number of residues per molecule when the molecular weight is more accurately determined or when the amino acid sequence of the protein is determined.

A final point of interest concerns the potential clinical utility of streptokinase in the treatment of thrombotic disease in man. Several of the highly purified preparations prepared by repetitive column chromatography on DEAE-cellulose under conditions shown in Figs. 2 to 6 and Table I have already been administered intravenously into man. The pioneer investigations of Sherry et al. (35), Alkjaersig, Fletcher, and Sherry (36), and Fletcher, Alkjaersig, and Sherry (37) are particularly noteworthy in this regard. These authors have carefully established conditions under which streptokinase may be infused to produce the activation in vivo of plasminogen and have defined the biochemical sequence in vivo which ensues following such infusion. They have also given a preliminary account of the effects of intravenously administered streptokinase in patients suffering from acute myocardial infarction (38). Significant also in this regard are the studies of Johnson and McCarty (39) who provided evidence for the dissolution of experimentally induced thrombi in man following streptokinase infusion. The work of Sherry et al. (35) and Johnson and McCarty (among others) should be consulted by those interested in the potential use of streptokinase in human therapy and in the problems associated with the administration of this bacterial protein in man.

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