Methods Employed for Purification of Streptokinase.* (22909)

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Tillett and Sherry(1) introduced the local use of streptokinase into human therapeutics following the description of a method for its partial purification(2). However, the clinical toxicity of the available preparations has been sufficient to preclude its systemic use in man except under restricted conditions(3).

The present communication describes methods for the further purification of streptokinase, and a further paper(4) reports the assay of these preparations in man for clinical toxicity. Though the purified preparations were much less toxic to man than the currently available preparations, activity/nitrogen ratios did not always parallel clinical toxicity. For this reason, alternative methods of purification are briefly described in addition to that finally adopted.

Materials and methods. The streptokinase assay was based on that of Christensen(5) with the modification that dilutions were made directly into each assay tube with a micro-burette.† This modification allowed the use of more closely spaced dilutions than were employed in the original procedure, and increased both speed and accuracy. Assays were expressed in terms of the Christensen unit(5), each assay being the average of duplicate determinations. If the duplicates were discrepant by more than 10%, assays were repeated until satisfactory agreement was obtained. The reproducibility of the assay method was assured by means of frequent control determinations on the National Institute of Health streptokinase standard. *Nitrogen* determinations were made by a standard micro-Kjeldahl method. In addition, optical density at 260 mμ and 280 mμ was followed with a DU Beckman spectrophotometer. *Starch zone electrophoresis* followed the procedure of Kunkel and Slater(6) except that a wider (20 cm) trough was used. Water, at 4-6°C, was pumped through hollow top and bottom plates to provide extra cooling. The buffer had the following composition: sodium borate 8.5 g, boric acid 0.68 g, water to 1 liter adjusted to pH 8.9. Owing to the fact that the borate ion interacts with the starch, the effective ionic strength could not be calculated. The starch‡ was prepared by washing 1 kg twice with 3 liters of 0.05 M acetic acid at 50°C, washing with 5 liters of 1% saline on a Buchner funnel, and then washing twice with 3 liter amounts of borate buffer. Five-six ml of 0.5-2% streptokinase was used for each electrophoresis run, which was made for 16-24 hours at 300-450 volts and 60-100 milliamps. The block was cut into ½-inch strips, each starch strip being placed in a sintered glass funnel and eluted with successive small volumes of 1% saline, containing 0.01 M phosphate buffer at pH 7.6, to a total of 15 ml. A Raymond-type apparatus(7) was used for electrophoresis-convection studies. Paper electrophoresis was performed with a horizontal type machine, using barbital buffer at pH 8.6 and ionic strength 0.1; amido-black stain was used. Streptodornase was determined by a viscosimetric method(8). *Fractionations* were performed at 2-5°C with aqueous solutions, and at −5 to 0°C with organic solvent-aqueous solutions. Saline refers to 0.155 M sodium chloride solution. Sintered glass filters were of medium porosity, unless otherwise stated. Apparatus was acid cleaned, and fractionation procedures were completed as rapidly as possible to prevent contamination with pyrogens. The starting material for fractionation was a lyophilised partially purified streptokinase-streptodornase concentrate.§

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† Micro-metric Instrument Co., Cleveland, O.

‡ Eimer and Amend, New York City.

§ Bulk Varidase® a commercial streptokinase-streptodornase preparation, prepared by a modified Christensen method(3), and kindly supplied by Mr. Frank Ablondi, Lederle Laboratories, Pearl River, N. Y.

Results. In this and the ensuing article(4) reference is made to 4 methods of streptokinase purification. The different methods were not substantial changes in technic, but usually represented additional steps taken beyond those of the previous procedure. For purposes of correlation between the biochemical purification procedures and the clinical effects of the material(4) the streptokinase preparations are specifically identified as methods 1, 2, 3, and 4. The starting material usually assayed at 85 units/gamma N, though 2 lots were 95 units gamma N. There were differences between lots obtained in bulk with regard to color, solubility, and the amount of insoluble residue. Despite these physical differences all 6 bulk lots used exhibited similar purification characteristics.

Method 1. The concentrate was taken up in versene®‖ (sodium ethylenediaminotetraacetate)-saline-phosphate, and clarified by sintered glass filtration. The filtrate was equilibrated with excess ether in the cold and then quick-frozen in a dry ice-alcohol mixture. Two hours later it was cautiously melted and the lipid layer centrifuged off. The clear aqueous phase was adjusted to pH 5.3 with 1 M acetate buffer and the streptokinase was precipitated with alcohol to a final concentration of 12.5%. The yield of streptokinase was found in different runs to vary between 33-52% of the original, the purity being 150-220 units/gamma N. Four zone electrophoretic runs performed with this material gave yields of 33-65% and potencies of 340-450 units/gamma N with a total of 1-2 million units in each batch. In these as in all the other preparations used in man, the streptodornase content was negligible, being less than 100 units of streptodornase for every million units of streptokinase. The final chemical purification method is described below.

Method 2. Ten g of streptokinase concentrate, 100 ml of 0.5 M versene and 1600 ml of 0.02 M phosphate-saline were adjusted to pH 9.0 with 0.1 N sodium hydroxide. The mixture was stirred until most of the material had dissolved, the pH was then lowered to 8.0 with 0.1 N hydrochloric acid. (Two batches were largely insoluble at pH 9.0, but dissolved when the pH was raised to 11.0 for 2-3 minutes.) Forty g of washed celite¶ was added, and after 30 minutes of stirring, the mixture was filtered through sintered glass. Four hundred g of calcium phosphate gel(9) was stirred with the filtrate for 1 hour, after which the gel was removed by centrifugation. 10-15% of the nitrogen was removed at this step with some 5% of the streptokinase activity, the filtrate changed from opalescent to clear, and the 260/280 optical density ratio fell from 0.76 to 0.64. The filtrate was brought to 30% ammonium sulphate saturation and after 3 hours was filtered through sintered glass using 5-10 g of celite as a filter aid. The precipitate was discarded, the filtrate brought to 42% ammonium sulphate saturation and left overnight. The precipitate was collected on a 30 cm Buchner funnel using No. 50 Whatman paper, it was washed with 200 ml of saline at 42% ammonium sulphate saturation. The precipitate and paper were eluted with 400 ml of saline containing 0.025 M tris (hydroxymethyl) aminomethane buffer at pH 8.0. The solution was readjusted to pH 8.0 with 0.1 N sodium hydroxide and filtered through sintered glass. The yield at this point was 45-60% of the starting activity, the purity usually 300 units/gamma N and the 260/280 optical density ratio 0.53. The filtrate was cooled to 0°C in a refrigerated tank and ether (precooled to −5°C, 50 ml per 100 ml of filtrate) was added with stirring over a period of 3 hours. The temperature was slowly lowered during this time until, at full ether saturation, it was −5°C. The mixture was centrifuged at −5°C and 400 × g for 1 hour in centrifuge separating funnels. The ether-aqueous layer was again equilibrated with ether (25 ml ether per 100 ml of solution) and centrifuged. The ether-aqueous layer (300-360 ml) was added to 500 ml of ether saturated distilled water containing 4 ml of 0.2 M sodium acetate. The pH was adjusted to 6.2 with 0.2 M acetic acid and sufficient 1 M zinc acetate

‖ Brand of sodium ethylenediaminotetraacetate, Bersworth Chemical Co., Framingham, Mass.

¶Analytical Celite, Johns-Mansville Products.

added to bring the zinc concentration to 0.01 M. Next morning the solution was centrifuged and the precipitate taken up in 15 ml of 0.25 M versene-saline. (The yield was 13-19 million units representing 15-20% of the original activity, the purity 370-430 units/gamma N and the 260/280 optical density ratio 0.52.)

Method 3. One batch of method 2 material was precipitated with 42% ammonium sulphate, dialyzed, and precipitated with zinc at 0.01 M concentration. Nearly half the streptokinase activity was lost at this step and the potency was only raised from 370 to 410 units/gamma N. However, in view of its different toxicity to man, this is referred to as *method 3.*

Method 4. Three lots of method 2 material were subjected to starch zone electrophoresis and streptokinase assaying at 515, 600 and 600 units/gamma N was obtained with a yield of 35-60%.

Characterization. The fractionation procedures were guided by paper electrophoresis. It was found that streptokinase moved with the mobility of an alpha 2 globulin in barbiturate buffer at pH 8.6. Unfortunately at purities of 400 units/gamma N and above, this procedure failed to provide useful information, as the material frequently ran as one component. Furthermore, material that had been subjected to starch zone electrophoresis sometimes showed more trailing than the original material of lesser potency.

Ultra-centrifuge and classical electrophoresis examination were made upon a sample of method 2 material, assaying at 430 units/gamma N, which ran as one component without detectable trailing on paper electrophoresis. The ultracentrifuge showed two components, the first was a single peak containing 88% of the sample with a sedimentation constant of 3.4 in pH 7.6 phosphate buffer of 0.1 ionic strength. The second component was of broad distribution with an approximate mean constant of 1.2. These figures can be converted to S_{20w} by adding 10%. The estimated molecular weight of the main peak was 50,000, and that of the second 5,000-25,000 with a mean of 15,000. Classical electrophoresis at pH 7.4 and pH 8.6 showed in each case 3 components: a slow component (4.7% of the material), a main peak (86.4% of the material) and a fast component (8.9% of the material). The respective mobilities at pH 8.6 and 10^{-5} cm sec/volts/cm were 1.3, 3.8, and 4.2 for the descending phase, and 2.4, 3.5, and 5.2 for the ascending phase. The streptokinase activity was contained in the main peak. It seems clear from the mobilities that the two minor components should be separated from the main peak on starch zone electrophoresis and, indeed, such a distribution was found. However, it would also appear from the consideration of the relative increase of potency obtained after zone electrophoresis (20-30%), that this method of separation also partly resolves the main peak into two components. Neither the material prepared by chemical fractionation, nor that subjected to electrophoresis were homogenous on immuno-chemical analysis. Examination by the technic of Ouchterlony(10) against rabbit antibody showed 5-7 bands for method 2 material (2 samples) though the antigenic structure was much weaker than that of the original concentrate. Electrophoresis of these two samples (method 4) further weakened the antigenic structure and resulted in the disappearance of two bands. Unfortunately, this extremely sensitive test, under these circumstances, could not be used to provide a quantitative estimate of the impurity present.

Stability. Material for stability testing was brought to 1% human albumin final concentration, sterilized by Seitz filtration (Swinney model) and maintained at refrigerator temperature. It was found that in high concentration (approximately 5×10^5 units/ml) the activity fell about 30% in 6 weeks.

Discussion. A 6-7 fold purification of the original material was achieved by the methods described. Immunochemical analysis showed that the material prepared by zone electrophoresis was impure. It is difficult to estimate the amount of impurity as the results of ultra-centrifuge and classical electrophoretic studies suggested the presence of approximately 13% of impurity before zone electrophoresis, yet zone electrophoresis usu-

ally gave a 20-30% increase in activity/nitrogen ratios. It is possible that mildly denatured material may have similar physical and chemical properties to the active substance. This conclusion was supported by the observation that if the biological activity of the material was allowed to decay at room temperature under sterile conditions its electrophoretic and chemical properties were apparently unchanged. Severer conditions did, of course, change these properties. Thus the tests of homogeneity used do not certainly distinguish between biologically active and biologically inactive protein. For this reason no estimate can be offered as to the biological potency of the "pure" streptokinase.

The purification methods, themselves, leave something to be desired. Particularly at the ether stage the manipulations are exacting and the most rigid temperature control is mandatory. Moreover, starch zone electrophoresis, with its inherently low capacity, is an unsuitable method for large-scale purification. It was hoped that this latter procedure could be replaced by electrophoresis-convection, but some 25 runs revealed that the only moderately satisfactory mode of operation was at the isoelectric point of streptokinase (pH 4.7-5.0), and here the solubility was so low that only small amounts of material could be handled. Though the present methods appear to be of an interim nature, they have sufficed to prepare enough material for clinical trial(4) and provide leads for better methods.

Summary. Chemical fractionation of a currently available, partially purified streptokinase-streptodornase concentrate yielded a 4-5 fold purification of the streptokinase. The addition of a starch zone electrophoresis to the methods increased the purification to 6-7 fold. Biophysical examination suggested that the protein was largely homogenous, but immunochemical analysis revealed the presence of extraneous antigenic components. Ultracentrifuge examination suggested that streptokinase has a molecular weight of approximately 50,000.

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