PHYSICAL AND IMMUNOLOGICAL DIFFERENCES AMONG STREPTOKINASES*

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Group A beta hemolytic streptococci have been shown to produce a number of extracellular products with widely varying biological activities (1). The relationship between infection by Group A streptococci and the subsequent development of either acute rheumatic fever or acute glomerulonephritis has stimulated much interest in these products. The fact that most of these products are antigenic and, following infection in man, result in the development of specific neutralizing antibody has formed the basis for several useful streptococcal antibody tests (2–4) and for a number of hypotheses as to the pathogenesis of these complications (5, 6).

Some progress has been made in separation of the extracellular products of Group A streptococci by zone electrophoresis and column chromatography (7–10). In this investigation, certain strains of Group A streptococci, known to produce several extracellular products, including desoxyribonucleases, streptolysin O, and streptokinase, were selected for further attempts at separation and purification of these products.

The present report presents evidence that there are physical and immunological differences between the streptokinases produced by certain Group A strains and compares these Group A streptokinases with streptokinase of Group C origin.

Materials and Methods

Strains of Streptococci.—Strains of Group A streptococci used in electrophoretic, chromatographic, and immunologic studies are as follows:

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Strain C-203 possesses a Type 3 M antigen and a Type 1 T antigen. This strain was kindly furnished by Dr. Rebecca Lancefield.

Strain 2184 is a Type 12 streptococcus, isolated from a patient with acute nephritis by Dr. Floyd Denny.

Strain H-105 is a variant of the CO strain of Tillett which was used for some of the original studies on streptokinase (11). It is an undesignated new type.

Strain 3539 is a Type 1 streptococcus, found to produce high yields of streptokinase. This strain was isolated from a patient at the University of Minnesota.

The following strains of Group A streptococci were employed in electrophoretic studies only:

Strain 4784 or A 236 (small colony) was isolated from a mouse epizootic as described by Bell *et al.* (12) and obtained from Dr. Rebecca Lancefield.

Strain 2465 or 49 is a Type 12 tetracycline-resistant strain obtained from Dr. W. J. Mogabgab.

Strain 2501 or 1 GL 88 is a Type 17 streptococcus, isolated at Great Lakes Naval Station from a patient with scarlet fever who subsequently developed acute rheumatic fever. This strain was obtained from Dr. Rebecca Lancefield.

Group A strains of streptococci used in immunologic studies only included six Type 1 strains, several Type 4 and Type 12 strains, and single strains of Type 3, Type 25, Type 28, and Type 49, all taken from stock strains among the collection in this laboratory.

A single strain of Group C streptococcus, strain H-46A, was used in electrophoretic and immunologic studies. This is the Group C strain used by Tillett (13) and by Christensen (14) for streptokinase studies and also used for commercial preparation of this extracellular product.

Preparation of Concentrates of Extracellular Products.—Strains of streptococci were grown in a dialysate medium of Pfanstiehl peptone, R.I., casamino acids, and beef heart infusion (8). Concentrates of the extracellular products in the supernatant fluid were prepared by precipitation with ammonium sulfate, filtration of the suspension through starch to collect the precipitate, and washing of the starch pad with glycine buffer to elute the precipitated protein for further concentration by filtration through a collodion bag (8). This technique was modified when it was found that higher yields of streptokinase are obtained if the filtered supernatant is refiltered through another starch pad after standing an additional 24 hours. The washings from this pad are then combined with the original eluate for concentration in collodion bags.

Electrophoresis.—Extracellular concentrates were examined by starch zone electrophoresis according to the method of Kunkel and Slater (15). Glycine buffer, $\mu = 0.1$, pH 9.0, was used. A direct current with a potential difference of 400 v was applied for 16 hours at 4°C. The protein was eluted from 1 cm sections of the starch block by washing with 2.0 ml of glycine buffer.

Column Chromatography.—Extracellular concentrates were separated by column chromatography with diethylaminoethyl cellulose (DEAE cellulose) as the ion exchange material. Selectacel, standard DEAE cellulose, lot 1326, obtained from Carl Schleicher and Schuell Co., Keene, New Hampshire, was used throughout these experiments. This material has a capacity in meq/gm of 0.87. The cellulose was prepared for use by the methods described by Peterson and Sober (16). Glycine buffer, $\mu = 0.01$, pH 9.0, was used as the final wash and suspending buffer in preparation of the cellulose. A 30 cm glass column, with a 1.0 cm inside diameter was used, and the cellulose was packed to a height of 15 cm. The proteins were separated by salt gradient elution. The starting buffer was glycine, $\mu = 0.01$, pH 9.0, and the second flask contained 1.0 M NaCl in the same glycine buffer. Flat bottom beakers containing 240 ml of the appropriate solution were employed to insure a linear gradient. An average of four fractions per hour, containing 2 ml each, were collected in each experiment.

Protein Determinations.—Protein was determined in the electrophoretic and chromatographic fractions by a modification of the Folin-Ciocalteu method (17).

Desoxyribonuclease Determinations.—Desoxyribonuclease (DNAse) activity was measured by an adaptation of the alcohol precipitation test (8, 18). The DNAse's utilized as markers in this study were identified immunologically by employing specific neutralizing antisera in a modification of the McCarty alcohol precipitation test (9, 18).

Streptokinase Determinations.—Streptokinase determinations were done by a modification of the method of Kaplan (19). Streptokinase dilutions were made in phosphate buffer, 0.1 M, pH 7.4, containing 0.5 per cent gelatin, prepared according to the method described by Christensen (14). The substrate solution used in streptokinase assays was a 0.4 per cent solution of fraction II, human plasma (human fibrinogen, parogen, prepared by Cutter Hospital Products, Berkeley, California). Two lots, G 4005 and G 3911, were used for these studies. Substrate dilutions were made with buffered saline. For assaying streptokinase activity, 0.1 ml aliquots of starch block fractions or column eluates were placed in a 10 x 75 mm test tube. To each tube 0.5 ml of substrate solution was added, and the mixture was clotted with 0.1 ml of thrombin solution (Parke, Davis and Co., Detroit) containing 10 units of bovine thrombin/ml. The clots were incubated for 30 minutes in a 37°C water-bath. The end-point was read as the highest dilution resulting in complete lysis of the standard clot.

Commercial Preparations of Streptokinase.—Varidase (lot 2200-968-A) is a commercial preparation prepared by Lederle Laboratories, Pearl River, New York, containing both streptokinase and streptodornase, of Group C streptococcal origin. Kinalysin (lot C1914) is a relatively purified preparation of Group C origin, prepared and kindly furnished by Merck, Sharp and Dohme Drug Co., West Point, Pennsylvania.

Examination of Streptokinase Preparations for Proteolytic Activity.—Streptokinase-containing fractions prepared in this laboratory and the commercial streptokinases were examined for their ability to lyse bovine fibrinogen clots. A single lot of Armour bovine fibrinogen, lot 55-127, 96.5 per cent clottable after further purification by Laki's method (20), kindly furnished by Dr. R. Von Korff, was used in these experiments. The test solutions of streptokinase were first standardized by determining their ability to lyse a standard human fibrinogen clot. Various concentrations of the streptokinase solutions were then employed, up to 1000-fold over the amount required to lyse a standard human fibrinogen clot. Two-tenths ml of a known solution of streptokinase was incorporated into a bovine fibrin clot, containing 0.5 ml of a 0.6 per cent fibrinogen solution clotted with 0.1 ml thrombin. Incubation was carried out in a water-bath at 37°C. Observations were made at hourly intervals for 8 hours and clots were allowed to stand in the water-bath overnight for observations after 24 hours of incubation.

The streptokinase preparations were also examined for evidence of proteolytic activity on heated and unheated bovine fibrin plates prepared by the method of Astrup and Mullertz (21) and kindly furnished by Dr. Henry Gans. In this experiment 0.05 ml of varying concentrations of the streptokinases under study were spotted on plates containing heated or unheated bovine fibrin and then were incubated 18 hours at 37°C. Control-heated plates were run simultaneously, in which human plasminogen had been added to the test spots. The human plasminogen was prepared in Dr. Gans' laboratory from plasma fraction III, furnished by E. R. Squibb and Sons, New York.

Studies of the Effect of pH and Temperature on the Stability of Streptokinase.—These experiments were done by a modification of the methods described by Christensen (22). The streptokinase solutions were incubated at 56°C for 1 hour in buffers of the appropriate pH. Acetate buffer was used for determining the effect of pH 5.1 and 5.5 and glycine buffer for pH 9.5. For assaying residual streptokinase activity, dilutions were made in phosphate-gelatin buffer, pH 7.6.

Inhibition of Plasminogen Activation by Epsilon Aminocaproic Acid (EACA).—The streptokinases being compared in these experiments were standardized as in the antibody studies. To insure streptokinase excess, the concentration of streptokinase used in the inhibition studies was twice the concentration resulting in complete clot lysis in 30 minutes. The stock

solution of epsilon aminocaproic acid (EACA) contained 20 mg/ml. Serial 2-fold dilutions were made from this stock solution. Streptokinase, 0.2 ml, and EACA dilutions, 0.1 ml, were mixed and allowed to stand at room temperature for 15 minutes. Then fibrinogen was added, and the clot was formed by addition of thrombin. Results were recorded as the minimal concentration of EACA resulting in complete inhibition of clot lysis by the various streptokinases being tested.

Preparation of Specific Rabbit Antisera.—Rabbits were immunized with streptokinase fractions from Group A strains prepared by starch block electrophoresis or by column chromatography. Commercial varidase or kinalysin was used as a source of streptokinase of Group C origin for immunization. An emulsion was prepared containing two parts antigen solution, two parts heavy mineral oil, and one part aquaphor. The mixture was allowed to cool and was injected subcutaneously in 2 ml volumes. Injections were given twice weekly. After the 1st month the animals were rested for a period of 4 weeks, and injections were then continued for a total of at least 2 additional months.

Streptokinase Antibody Determinations.—These determinations were done by a modification of the method of Kaplan (19). Streptokinase from Group A strains used in antibody determinations consisted of partially purified fractions prepared by either starch block electrophoresis or column chromatography techniques. In some instances, lyophilized preparations were redissolved in buffer and in other instances freshly prepared antigens were employed. The streptokinase of Group C origin used in antibody assays was either varidase or kinalysin, both of which are commercial preparations. Gelatin buffer, as prepared for the streptokinase assay was used in making streptokinase dilutions. For most of the antibody determinations, Cutter human fibrinogen, lot G 4005 was used. However, when a second lot, G 3911, was employed, using known antisera as controls, no significant differences in titers were obtained.

Immunoelectrophoresis.—Microslide immunoelectrophoresis was done by methods described by Crowle (23). Agar-agar, (Baltimore Biological Laboratories, Baltimore, lot 111623) 1.5 per cent, was used in preparing slides. A barbital buffer, $\mu = 0.1$, pH 8.2, was employed. Electrophoresis was conducted at 4°C for 1 hour with a potential difference of 60 v. Photographs were taken of unstained slides.

Antistreptolysin O Determinations.—These determinations were done by the method of Rantz and Randall (24).

RESULTS

A difference in the mobilities of the streptokinases from different Group A strains was initially suggested when individual electrophoretic patterns of extracellular products from strains 2184 and C-203 were examined. The production of similar desoxyribonucleases (DNAse's) by these two strains made it possible to utilize these enzymes as markers in comparing the mobilities of the streptokinases. The DNAse D of strain 2184 was observed to have the same mobility as that of strain C-203, and this enzyme was of particular value as a marker. Under the conditions of this study, the peak activity of DNAse D of strain C-203 coincided with the peak activity of streptokinase produced by that strain, but the streptokinase of strain 2184 migrated further towards the anode.

In order to exclude the possibility that minor variations in physical conditions of individual experiments might result in these differences in mobility of streptokinase, concentrates of the extracellular products from these two

strains were separated in parallel starch blocks in the same experiment and artificial mixtures of the extracellular products from the two strains were also examined by electrophoresis. The results of the parallel starch block experiment are shown in Fig. 1. The upper portion of the graph illustrates the electrophoretic distribution of the streptokinase and desoxyribonucleases of strain 2184. The lower section of the graph represents the pattern obtained with the extracellular concentrate of strain C-203. It can be seen that the peak activities of DNAse B and DNAse D of these two strains are located in the same zones of their respective starch blocks. Strain C-203 is also observed to produce a

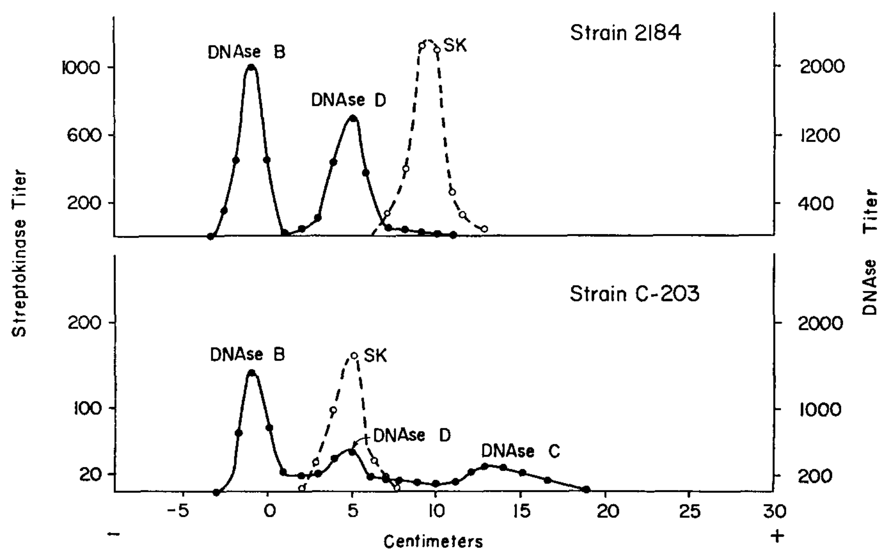

Fig. 1. Comparison of the distribution of extracellular products of strain 2184 and strain C-203 by parallel zone electrophoresis. (SK, streptokinase, DNAse, desoxyribonuclease.)

small quantity of DNAse C. The streptokinase of strain C-203 shares the same zone as DNAse D of that strain, while the streptokinase of strain 2184 has migrated more towards the anode, and its peak is separated by about 5 cm from the peak of the DNAse D activity.

Resolution of an artificial mixture of the extracellular concentrates of these two strains by zone electrophoresis is illustrated in Fig. 2. In this experiment, streptokinase activity resolved into two distinct peaks separated by approximately 9 cm. Desoxyribonuclease peaks were typical in distribution. The streptokinase peak nearest the point of origin coincided with the DNAse D peak of this mixture and was compatible with the previously observed mobility of streptokinase of strain C-203. The second streptokinase peak was more anodal in its mobility and was well separated from the DNAse D peak as was

previously observed with the streptokinase of strain 2184. As will be subsequently shown, the two streptokinase peaks could be identified serologically as representing the streptokinases of strains C-203 and 2184 respectively.

Evidence of physical differences between the streptokinases of these two strains was also obtained by column chromatography. Salt gradient elution from a diethylaminoethyl cellulose (DEAE cellulose) column produced excellent separation of the DNAse's, and these again proved to be useful markers in comparing the streptokinases of these two strains. In individual experiments, performed under the same conditions, the streptokinase of strain C-203

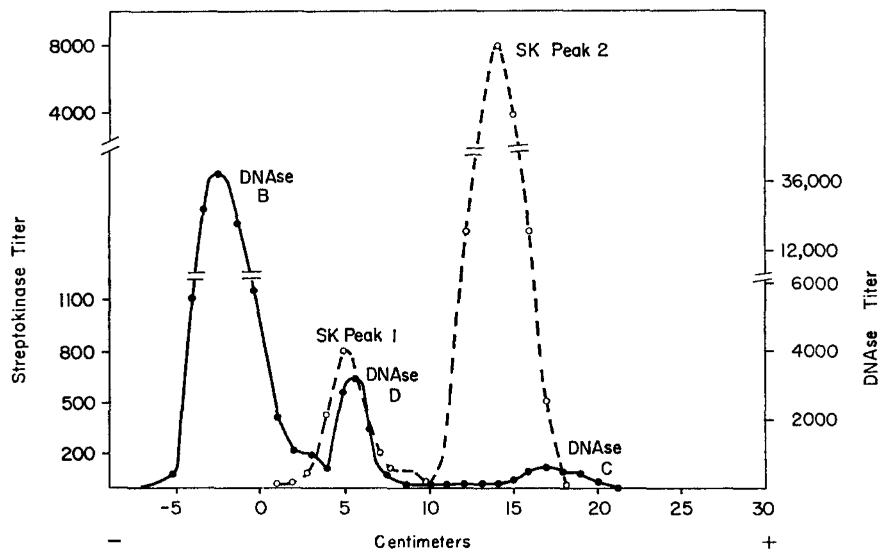

Fig. 2. Resolution by zone electrophoresis of an artificial mixture of the extracellular products of strains 2184 and C-203. (SK, streptokinase, DNAse, desoxyribonuclease.)

was eluted in the same fractions as the DNAse D of this strain, while the streptokinase of strain 2184 was eluted at a higher salt concentration than the DNAse D of that strain. When a mixture of the extracellular products from these two strains was applied to a DEAE cellulose column, excellent resolution of the two streptokinases was obtained (Fig. 3). The first streptokinase peak to be eluted was identified in the same fractions as the DNAse D of this mixture and thus appeared to correspond with the streptokinase peak obtained when strain C-203 was chromatographed separately. The second streptokinase peak was well separated from DNAse D activity and was eluted at a salt concentration comparable to that of strain 2184 streptokinase. Subsequent serologic examination of the two streptokinase peaks identified them as the streptokinases of strains C-203 and 2184, respectively. Similar elution patterns were

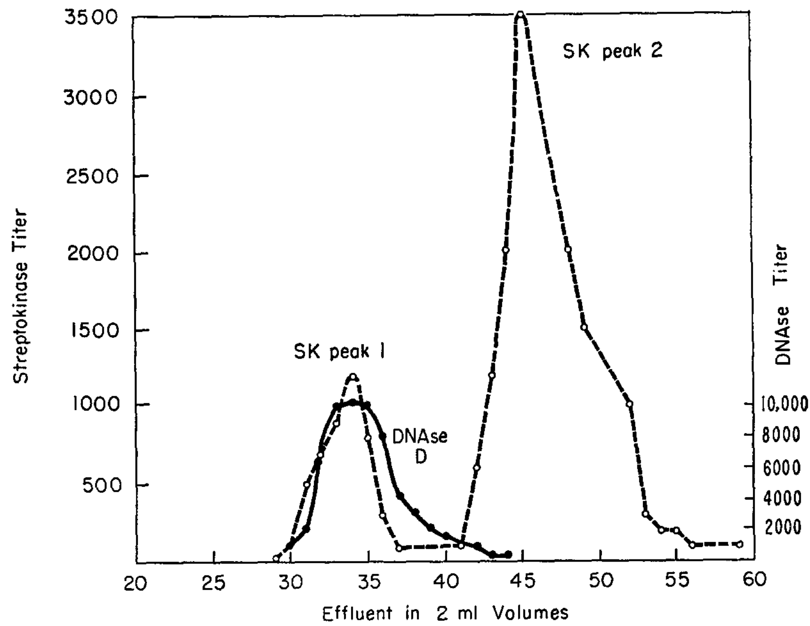

Fig. 3. Resolution by column chromatography of an artificial mixture of the extracellular products of strains 2184 and C-203. (SK, streptokinase, DNAse, desoxyribonuclease.)

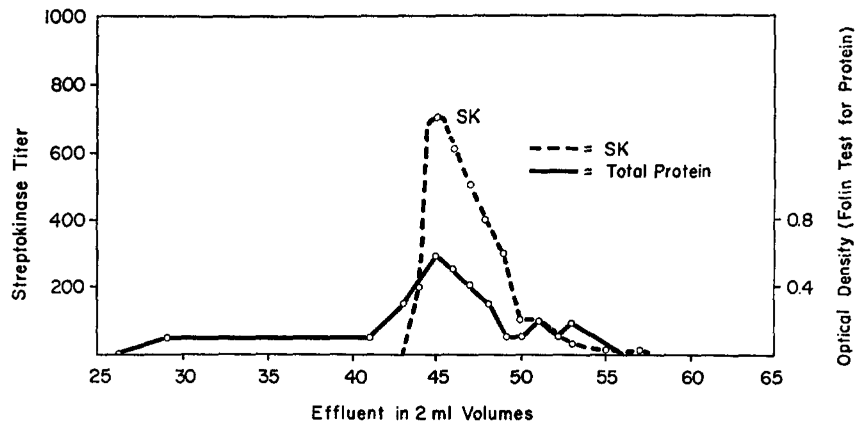

Fig. 4. Rechromatography of streptokinase peak 2 from Fig. 3. (SK, streptokinase.)

obtained on rechromatography. Fig. 4 illustrates the results of rechromatography of the streptokinase from the second peak shown in Fig. 3, the peak corresponding to the streptokinase of strain 2184. On rechromatography, a single peak of streptokinase activity was obtained and this was eluted at the

same salt concentration as in the original mixture. The streptokinase obtained in this peak was subsequently shown to be immunologically like that of strain 2184 streptokinase.

Although the culture conditions employed do not favor proteinase production, the possibility that one of these activities might represent proteinase rather than streptokinase activity was considered. This possibility was examined by determining the ability of these materials to lyse bovine fibrinogen clots, which are susceptible to the former but not to the latter product. No clot lysis occurred with any of the streptokinase preparations being investigated, even though concentrations up to 1000-fold over the amount required to lyse a standard human fibrinogen clot were employed and observations were continued over a 24 hour period.

TABLE I

Immunological Comparison of Streptokinases from Two Group A Strains: Neutralizing Titers with Homologous and Heterologous Antisera

Antigens	Antisera	
	Anti-2184 SK	Anti-C-203 SK
2184 SK (SK-A)*	1:417	1:83
C-203 SK (SK-B)‡	1:83	1:417
No. of tubes difference in titer	4	4

* Streptokinase from strain 2184, subsequently referred to as SK-A.

‡ Streptokinase from strain C-203, subsequently referred to as SK-B.

Similarly, evidence for proteolytic activity was sought by testing the streptokinases for their ability to lyse heated and unheated bovine fibrin plates. No proteolysis occurred on either the heated or unheated plates. With the addition of a preparation of human plasminogen to the heated fibrin plates, all of the streptokinase preparations produced definite proteolysis. With a constant amount of plasminogen the extent of the proteolysis was related to the concentration of streptokinase employed. These findings indicated that the individual peaks obtained on electrophoresis and column chromatography were due to activation of plasminogen and not to proteinase activity.

Upon the demonstration of distinct physical differences in the streptokinases of the two strains, 2184 and C-203, the possibility of immunological differences in the streptokinases from these two strains was investigated. Lyophilized fractions of streptokinase from strains 2184 and C-203, obtained by electrophoretic or chromatographic separations, were used to immunize rabbits. The differences in titer obtained when antisera to the streptokinase of strains 2184 and C-203 were examined with the homologous and heterologous antigen are presented in Table I. Although some degree of cross-reaction was observed,

significantly higher titers were obtained with the homologous systems, suggesting at least partial antigenic specificity. These findings were consistent with all preparations of antigen and antisera examined, although serum from earlier bleedings during the course of immunization usually showed lower titers and less cross-reactivity. These antisera were sufficiently specific to be useful in immunological identification of the individual peaks of streptokinase obtained by electrophoresis or by column chromatography. For convenience of discussion, the streptokinase of strain 2184 is subsequently referred to as SK-A and that of strain C-203 as SK-B.

A comparison was made of the physical and immunological properties of these streptokinases with those obtained from other sources. The streptokinase produced by another Group A strain, H-105, was shown to be similar to SK-A. Both the electrophoretic distribution and the chromatographic elution of this streptokinase were quite similar to those patterns characteristic of SK-A. Immunological studies, utilizing H-105 streptokinase and specific antisera to this material, in comparison with the antigens and antisera of SK-A and SK-B, indicated that the streptokinase of strain H-105 was also immunologically like that of SK-A.

The electrophoretic mobility of the streptokinases of Group A strains 2501, 2465, and 4784 was found to be similar to that of SK-A. Immunologic studies have not been done with the streptokinases of these strains.

The streptokinase of a Type 1 strain, 3539, an unusually good producer of streptokinase, was found to be electrophoretically and immunologically similar to that of strain C-203 (SK-B). As with the latter strain, when a concentrate of the extracellular products of strain 3539 was mixed with a concentrate from strain 2184, good resolution of the streptokinases of these two strains was obtained by column chromatography. Antisera to SK-A and SK-B were employed in confirming the immunological identities of the two peaks. Better separation of streptokinase and DNAse D was obtained by chromatography of extracellular preparations of strain 3539 than with preparations of strain C-203, apparently due to elution of the streptokinase of the former strain at a slightly lower salt concentration.

Antisera to SK-A and SK-B have been employed in the screening of crude supernates of a number of Group A strains of streptococci, other than those strains examined by electrophoresis or chromatography. The strains examined included six Type 1 streptococci, several strains of Type 12 and Type 4 streptococci, and single strains of Type 3, Type 25, Type 28, and Type 49 streptococci. Neutralization patterns of the Type 1 and Type 3 strains suggest that these strains produce streptokinase immunologically similar to that of strain C-203 (SK-B) while the streptokinase obtained from strains of other types were immunologically like that of strain 2184 (SK-A).

Six human sera, with elevated antistreptolysin O titers, were employed in

TABLE II

Immunological Comparison of Group A Streptokinases: Neutralizing Antibody Titers in Human Sera Using Two Different Antigens

Antigens	Patient sera					
	J. K.	C. N.	S. C.	M. S.	L. F.	C. H.
SK-A*.	1:179	1:900	1:417	1:417	1:125	1:25
SK-B‡.	<1:25	1:900	1:417	1:125	1:125	1:36
No. of tubes difference in titer.	5	0	0	3	0	1
ASO titer§.	1250	1250	1250	833	1250	833

* SK-A obtained from strain 2184.
‡ SK-B obtained from strain C-203.
§ Antistreptolysin O titer (Todd units).

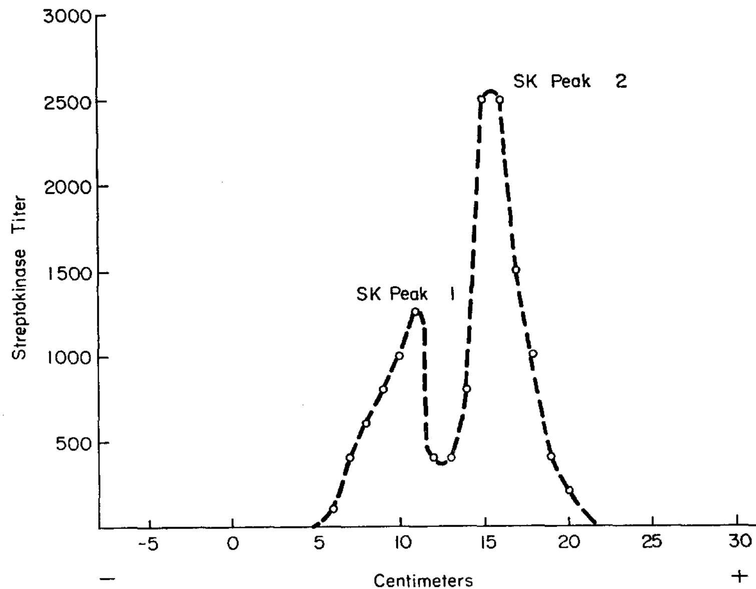

Fig. 5. Resolution by zone electrophoresis of an artificial mixture of the extracellular products of strain 2184 and a concentrated solution of varidase (Group C SK). (SK, streptokinase.)

streptokinase neutralization studies. A comparison was made of the titers obtained when SK-A and SK-B were used as antigens (Table II). The sera from two patients, J. K. and M. S., showed a significant difference when the two streptokinase antigens were compared.

Since much of the work previously reported has been done with commercial

TABLE III

Immunological Comparison of Streptokinase from a Group A Strain (C-203) and Commercial Kinalysin (Group C Streptokinase): Neutralizing Antibody Titer with Homologous and Heterologous Antisera

Antigens	Antisera	
	Antikinalysin	Anti-C-203 SK
C-203 SK (SK-B)	1:83	1:417
Group C streptokinase	1:179	1:56
No. of tubes difference in titer	2	5

TABLE IV

Immunological Comparison of Streptokinase from a Group A Strain (2184) and Kinalysin (Group C Streptokinase): Neutralizing Antibody Titer with Homologous and Heterologous Antisera

Antigens	Antisera	
	Anti-2184 SK	Antikinalysin
2184 SK (SK-A)	1:417	1:125
Group C streptokinase	1:278	1:179
No. of tubes difference in titer	1	1

TABLE V

Immunologic Relationships of SK-A, SK-B, and Group C Streptokinase Based on Neutralizing Antibody Determinations with Homologous and Heterologous Antisera

Antigen	Antisera		
	Anti-SK-A	Anti-SK-B	Anti-Group C streptokinase
SK-A	417*	83	125
SK-B	83	417	83
Group C streptokinase	278	56	179

* Reciprocal of neutralizing antibody titer.

streptokinase of Group C origin, it was desirable to compare this material with the Group A streptokinases under examination. Studies were performed on three Group C preparations; commercial varidase, commercial kinalysin, and an extracellular concentrate of their source strain, H-46A, prepared in this laboratory in the same manner as the Group A concentrates. The electrophoretic mobility in starch of these Group C preparations was found to be identical and was intermediate between that of SK-A and SK-B.

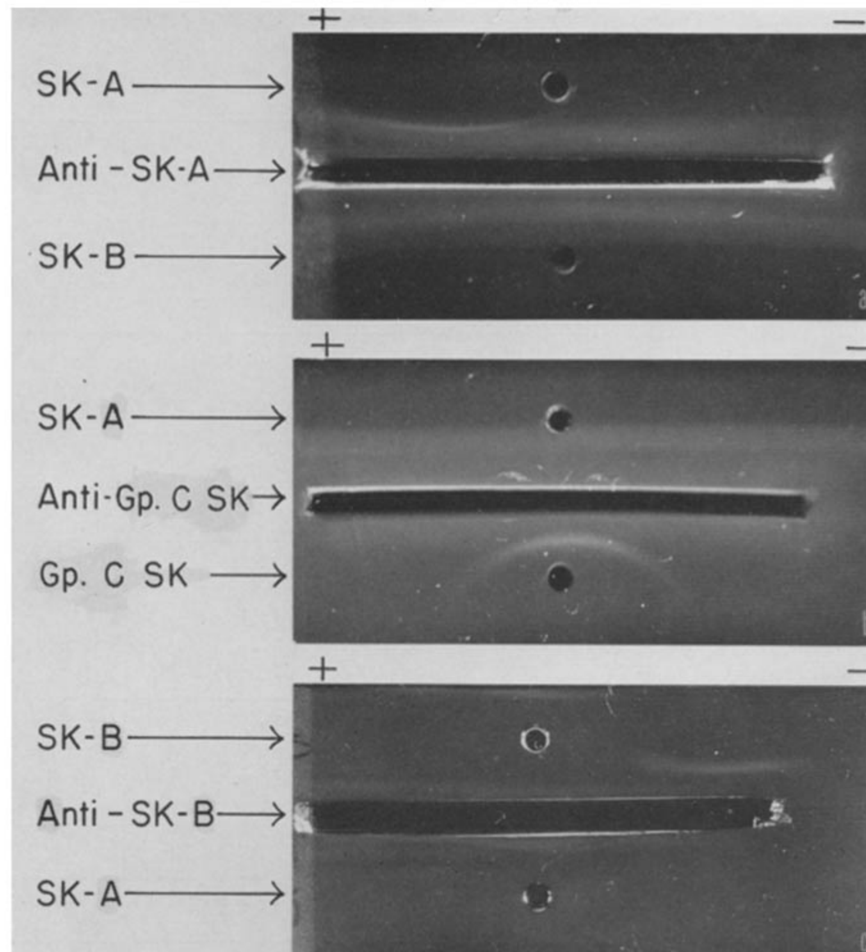

FIG. 6. (*a*) Immunoelectrophoresis of SK-A (top well) and SK-B (bottom well) with anti-SK-A (serum trench). Precipitin line with homologous antigen only. (*b*) Immunoelectrophoresis of SK-A (top well) and Group C streptokinase (bottom well) with anti-Group C streptokinase (serum trench). Precipitin line with homologous antigen only. (*c*) Immunoelectrophoresis of SK-B (top well) and SK-A (bottom well) with anti-SK-B (serum trench). Precipitin line with homologous antigen only.

Because of the immunological similarity of the Group C streptokinase to SK-A, subsequently shown, it was important to confirm the physical differences between these two streptokinases. Accordingly, an artificial mixture of an extracellular concentrate of strain 2184 (source strain for SK-A) and a concentrated solution of varidase were examined by starch zone electrophoresis.

Streptokinase activity partially resolved into two distinct peaks separated by 5 cm, illustrated in Fig. 5. Peak 1 had a mobility characteristic of the streptokinase of Group C (varidase) origin, and peak 2 had a mobility characteristic of SK-A (strain 2184).

Both kinalysin and varidase were used in the preparation of antisera to the Group C streptokinase and were found to be immunologically identical. The titers obtained when antibody to kinalysin and antibody to SK-B (C-203) were examined with homologous and heterologous antigens are illustrated in Table III. Although cross-reactions were noted, there appeared to be definite immunological differences. In Table IV are shown the data comparing SK-A and kinalysin. No definite immunological differences were found between these two antigens. Table V summarizes the immunologic relationships, based on

TABLE VI

Immunological Relationships of Three Streptokinases on Immunoelectrophoresis Using Concentrated Antisera

Antigens	Antisera		
	Anti-SK-A	Anti-SK-B	Anti-Group C streptokinase
SK-A	+++	0	0
SK-B	0	+++	0
Group C streptokinase	+	+	+++

+++, Strong precipitin reaction.
0, No precipitin reaction.
+, Definite precipitin reaction but weaker than seen with homologous antigen.

neutralizing antibody determinations, of SK-A, SK-B, and Group C streptokinase.

Immunoelectrophoresis has been used as another method for comparing SK-A, SK-B, and Group C streptokinase. The results of these immunoelectrophoretic studies confirm both the physical and immunological differences in these three streptokinases. The relative migrations of the streptokinases are similar to those observed when they are examined by starch zone electrophoresis. SK-A migrates towards the anode, SK-B moves toward the cathode, and the Group C streptokinase is intermediate between the two Group A streptokinases. These patterns are illustrated in Fig. 6. The cross-reactions seen in the neutralizing antibody studies do not appear in the immunoelectrophoresis experiments illustrated. This may reflect intrinsic differences in precipitating and neutralizing antibody to the streptokinases or may simply be a result of the concentrations of antigen and antibody used. The latter possibility is suggested by the fact that if 4-fold concentrated antisera to either SK-A or SK-B

were employed, a precipitin line would form with Group C streptokinase. On the other hand, even with concentrated antiserum to Group C streptokinase, precipitin lines with SK-A and SK-B did not occur. Similarly, use of concentrated antisera did not disclose cross-reactions between the SK-A and SK-B systems. These results are summarized in Table VI.

TABLE VII

The Effect of pH and Temperature on Streptokinase Activity

Strepto-kinases	Buffer	pH	Time	Temp.	Residual streptokinase activity at various test dilutions								
					1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800	1:25,600
			hr.	*°C*									
SK-A	Gelatin	7.6	1	Room	+	+	+	+	+	+	+	0	0
	Gelatin	7.6		56	+	+	+	+	+	+	+	0	0
	Acetate	5.1		56	0	0	0	0	0	0	0	0	0
	Acetate	5.5		56	+	+	+	+	0	0	0	0	0
	Glycine	9.5		56	+	+	+	0	0	0	0	0	0
SK-B	Gelatin	7.6	1	Room	+	+	+	+	+	+	+	+	0
	Gelatin	7.6		56	+	+	+	+	+	+	+	+	0
	Acetate	5.1		56	0	0	0	0	0	0	0	0	0
	Acetate	5.5		56	+	+	+	+	0	0	0	0	0
	Glycine	9.5		56	+	+	+	+	+	0	0	0	0
Group *C SK*	Gelatin	7.6	1	Room	+	+	+	+	+	+	+	0	0
	Gelatin	7.6		56	+	+	+	+	+	+	0	0	0
	Acetate	5.1		56	0	0	0	0	0	0	0	0	0
	Acetate	5.5		56	+	+	+	0	0	0	0	0	0
	Glycine	9.5		56	+	+	+	0	0	0	0	0	0

+, clot lysis.
0, no clot lysis.
SK, streptokinase.

The effect of temperature at various hydrogen-ion concentrations on the stability of SK-A, SK-B and Group C streptokinase was studied (Table VII). No significant difference in the stability of the three streptokinases was found. Each of the three streptokinases showed complete inactivation following incubation at 56°C. for 1 hour at pH 5.1. There was some loss of activity following incubation at pH 9.5, but all retained significant activity. Incubation at 56°C. at pH 7.6 did not result in loss of activity.

These several streptokinases were also compared in relationship to the inhibition of their plasminogen-activation by epsilon aminocaproic acid (EACA). The results are shown in Table VIII. These assays were carried out

simultaneously, employing varying concentrations of EACA with constant equivalent concentrations of streptokinase. There were no significant quantitative differences in EACA-inhibition of plasminogen-activation by any of the streptokinases. Although the use of this inhibitor failed to demonstrate any additional differences in these streptokinases, the fact that EACA in the expected concentrations did inhibit all of them was further evidence that the mechanism of induction of clot lysis by these streptokinases was the same, namely that of activation of plasminogen rather than direct proteolytic action.

TABLE VIII

The Effect of Inhibition of Plasminogen Activation of Streptokinases by Epsilon Aminocaproic Acid

Streptokinases	Epsilon aminocaproic acid concentrations (mcg/ml)							
	2000	1000	500	250	125	62.5	31.25	15.6
SK-A	0	0	0	0	0	+	+	+
SK-B	0	0	0	0	+	+	+	+
Group C	0	0	0	0	0	+	+	+

+, complete clot lysis.
0, no clot lysis.

DISCUSSION

Previous reports concerning the similarity or dissimilarity among streptokinases produced by different serologic groups of beta hemolytic streptococci, or by various strains of Group A streptococci, have been based on immunologic studies employing human sera. The most recent and best quantitated investigations are those of Kaplan (25) and Weinstein (26).

In Kaplan's studies of streptokinases from a Group A, a Group C, and a Group G streptococcus, in which sera from patients with infections caused by streptococci of the above-mentioned groups were used, there were no demonstrable immunological differences among these three streptokinases. The sera obtained from patients with Group C and Group G infections were of low titer. However, the sera from the Group A infections were of good titer, and did not differ in neutralizing titer when the three streptokinases from different serologic groups were used in antistreptokinase determinations. Kaplan concluded that there were no immunologic differences among streptokinases. It is probable that the currently accepted use of the commercial Group C streptokinase as the antigen in antistreptokinase determinations is based on Kaplan's studies.

Weinstein, employing Kaplan's methods for antistreptokinase determinations, studied the immunologic patterns of streptokinases obtained from 10 different strains of Group A streptococci. Using 25 sera obtained from patients with scarlet fever, he found that neutralizing titers differed significantly when the various streptokinases

from the 10 different strains were employed as antigens. He concluded that there was a definite dissimilarity in the streptokinases obtained from different Group A strains and considered the antistreptokinase test employing a single antigen as unreliable for this reason.

In an earlier study of "antifibrinolysins," Mote *et al.* (27) found results similar to those of Weinstein. Although handicapped by methods which were less quantitative than Kaplan's, these investigators found that titers varied when different strains of streptococci were used as the streptokinase source. This early study of Mote *et al.* and the later, more quantitative study of Weinstein thus support the evidence presented here that there are immunological differences in streptokinases obtained from different strains of Group A streptococci.

The present investigation indicates that there are both physical and immunological differences in the streptokinases produced by various Group A strains. The partial immunological cross-reactions that occurred suggest that there are antigenic relationships between these streptokinases, probably reflecting shared antigenic determinants. The possibility that these cross-reactions might represent contamination with small amounts of heterologous antigen cannot be excluded. However, this seems unlikely since in the studies of individual strains only single peaks of streptokinase were obtained and artificial mixtures of the streptokinases from different strains could be separated reasonably well by either starch block electrophoresis or column chromatography. In addition, antisera were prepared with the peak fractions of electrophoretic or chromatographically purified preparations which would tend to remove small amounts of contaminating antigens which might be undetectable in assay procedures. The partial cross-reactions seen between SK-A and SK-B began to appear as continued immunization of animals resulted in the development of antistreptokinase titers of 1:83 or greater with the homologous antigen. In two of the six human sera examined with SK-A and SK-B, definite differences in antistreptokinase titer were obtained. In the case of patient J. K., who had acute glomerulonephritis, it would appear that the child had had no experience with a streptococcus producing SK-B, and had not developed a sufficient antibody titer to SK-A for cross-reactions with SK-B to become apparent. The results with this patient's serum may thus be analogous to the results obtained when antibody titers to SK-A and SK-B were compared in sera from early bleedings of immunized animals.

Examination of these two streptokinases by immunoelectrophoretic techniques confirms the electrophoretic and neutralizing antibody studies indicating their differences. Precipitin lines occurred with the homologous antigen-antibody systems of SK-A and SK-B only and distinct differences in migration were apparent. It appears that this may be a useful method of demonstrating the immunologic differences that exist between these two streptokinases since, in the concentrations employed, the cross-reactions seen with the neutralizing antibody studies were not seen with this method.

Of a total of seven strains of Group A streptococci examined electrophoretically, five proved to have streptokinases with electrophoretic mobilities similar to that of SK-A while only two, C-203 and 3539, were observed to have the electrophoretic pattern characteristic of SK-B. It is of interest that these two strains also produced identical desoxyribonucleases. The similarity of the streptokinases of these strains suggested by electrophoretic studies could also be demonstrated immunologically.

In addition to those strains studied by electrophoresis, the streptokinases of a number of strains of various serologic types were examined for their immunologic relationship to either SK-A or SK-B by utilizing their crude supernates as antigens in neutralizing antibody studies. In these studies, only the streptokinases from Type 1 and the Type 3 strains were found to resemble SK-B. The remaining strains, including several of Type 12, a Type 4, a Type 25, a Type 28, and a Type 49, were indistinguishable from SK-A. The combined data of the electrophoretic and immunological studies clearly suggest that of the two Group A streptokinases represented by the prototype strains, SK-A appears to be the one more commonly encountered among Group A strains.

There is a possible relationship between serologic type of Group A streptococcus and production of either SK-A or SK-B. Thus, the only strains examined during this investigation with electrophoretic patterns typical of SK-B were the C-203 strain, a Type 3 streptococcus with a Type 1 T antigen, and 3539, a Type 1 streptococcus. Moreover, the only strains encountered during the screening of crude supernates containing streptokinase activity which were immunologically like SK-B were several Type 1 strains and a Type 3 strain. Earlier studies by the Commission on Acute Respiratory Diseases (28) demonstrated that patients with streptococcal infection due to Type 1 or Type 3 streptococci had a generally lower antistreptokinase titer than patients with certain other serologic types. They related the development of antibody formation in these patients to the *in vitro* production of streptokinase by the various serologic types isolated. Their studies indicated a difference in the median titers of streptokinase produced by various serologic types of streptococci and also demonstrated that frequency of production of streptokinase could be related to serologic type. It seems possible that some of the differences in antistreptokinase titers in the patients reported by this group might well be explained by differences in antigenic structure of streptokinases from strains of different serologic types.

Because of its extensive use as a prototype in studies of streptokinase and antistreptokinase and because of its use in clinical medicine, the streptokinase obtained from strain H-46A, a Group C streptococcus, which is the source of the commercial streptokinases, is of particular interest. The electrophoretic mobility of this Group C streptokinase was found to be intermediate between that of SK-A and SK-B. Although cross-reactions were observed, neutralization studies suggest that there is a definite quantitative immunological dif-

ference between Group C streptokinase and SK-B. Little or no difference between Group C streptokinase and SK-A was seen in neutralization studies. Immunoelectrophoretic studies revealed what appears to be only a one way cross-reaction between streptokinase of Group C origin and both Group A streptokinases which was dependent on the use of concentrated antisera to the latter.

The results of the neutralizing antibody studies are not necessarily in conflict with Kaplan's findings of immunologic similarity between streptokinases of Group A and Group C origin, since Kaplan examined the streptokinase of only one Group A strain, and since there appears to be little difference immunologically between one of the Group A streptokinases studied (SK-A) and the commercial Group C streptokinase. Indeed, since SK-A appears to be the more commonly encountered streptokinase of Group A origin, the selection of a single strain of Group A streptococcus for producing streptokinase would likely result in choosing a strain which elaborates SK-A.

The fundamental significance of the differences in streptokinases produced by Group A strains of streptococci and by other serologic groups of streptococi is not known. From a practical viewpoint, it is obvious that these differences may result in the erroneous interpretation of antistreptokinase data, since the antigen chosen for use in the antibody determination may differ from that produced by the infecting streptococcus. Immunological differences in streptokinases may also be of importance in regard to fibrinolytic therapy. Fletcher *et al.* (29) have shown that patients receiving streptokinase therapeutically may develop high antibody titers rather quickly and this may then interfere with streptokinase-activation of plasminogen. Streptokinase differing in antigenic structure from that customarily used in therapy might make prolonged therapy more feasible.

Differences in the physical and antigenic characteristics of streptokinases are also of interest from the standpoint of the biology of the streptococcus. The versatility of this organism in producing a variety of extracellular products is already well appreciated. The differences in streptokinases as represented by SK-A, SK-B, and Group C streptokinase have not been clarified beyond the fact that certain physical and immunological differences do exist. Electrophoretic mobilities and serologic data suggest that these differences in streptokinases may be somewhat analogous to the differences observed in the enzyme hybrids of the lactic dehydrogenase series of the developing chick (30). In this regard, it is of interest that the streptokinases, like the lactic dehydrogenases, show immunological cross-reactions, as might be anticipated in a series of hybrid molecules (30). This is in contrast to the streptococcal desoxyribonucleases which do not show immunological cross-reactions and therefore may not be true isozymes of the classical type. It is also of interest that most strains of Group A streptococci produce several different desoxyribonucleases, whereas

among the strains of Group A streptococci examined, each produces only one streptokinase. This characteristic in regard to streptokinase production facilitates immunological screening since this can be readily done with crude supernates.

Further definition of the differences in streptokinases may be expedited by the isolation of streptokinases in a more purified state. In addition, the development of a system for assaying streptokinase directly would simplify the comparison of preparations from different strains. Such knowledge concerning streptokinase would also contribute to the understanding of whether streptokinase can be considered a true enzyme rather than an activator substance (31).

SUMMARY

Two strains of Group A streptococci have been shown to produce streptokinases with different physical and immunologic characteristics. These streptokinases have been designated SK-A and SK-B. Examination of additional strains of Group A streptococci has indicated that SK-A is the more commonly encountered streptokinase.

Studies comparing the two Group A streptokinases with streptokinase of Group C origin have indicated that the latter streptokinase is intermediate between SK-A and SK-B in regard to its electrophoretic mobility. Immunological data suggest that the Group C streptokinase differs from SK-B but is closely related to SK-A.

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