

METHODS FOR MEASURING THE ACTIVITY OF COMPONENTS OF THE STREPTOCOCCAL FIBRINOLYTIC SYSTEM, AND STREPTOCOCCAL DESOXYRIBONUCLEASE¹

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Studies of the effects of streptokinase and desoxyribonuclease from hemolytic streptococci on certain pathological processes, reported in an accompanying paper (1), necessitated the development of quantitative methods suitable for routine laboratory assay of the various substances known to affect the streptokinase-plasminogen (streptococcal fibrin-lysing) system. Methods were devised, or earlier methods modified, for the routine assay of streptokinase (streptococcal fibrinolysin), antistreptokinase (antifibrinolysin), serum inhibitor (serum trypsin inhibitor), and plasminogen (serum protease, serum fibrinolysin, serum tryp-tase).

MATERIALS

1. *Crude streptokinase*:² The method used for production of streptokinase is based on one previously published (2), but differs principally in the use of ethanol as a precipitant rather than ammonium sulfate.

Cultures of Group C strain H46A are grown in the presence of high glucose concentration, employing intermittent neutralization with NaOH in the manner previously reported (2). The yields of streptokinase and desoxyribonuclease from a typical culture are presented in Figure 1. It will be noted that the peaks of enzyme production coincide with the peak of glucose utilization. As incubation is continued, both streptokinase and desoxyribonuclease levels decline. For this reason it is best to begin harvesting of the culture shortly before glucose utilization ceases.

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² We are greatly indebted to Dr. Benjamin Carey and Mr. J. N. Adam, Jr. of the Lederle Laboratories Division of the American Cyanamid Company for supplying large quantities of crude streptokinase for these studies.

At the end of the growth period the cells are removed by Sharples centrifugation at about 40,000 rpm. The supernatant is adjusted to pH 3.8–4.2 by the addition of glacial acetic or hydrochloric acid. Cold ethanol at -8°C is added to the supernatant at room temperature to a final concentration of 40–50%. After standing overnight in the cold, the practically clear supernatant is siphoned off and the flocculent precipitate, which contains the streptokinase and desoxyribonuclease, is collected by centrifugation at 2,000–3,000 rpm. The precipitate is dissolved in borate buffer with the aid of alkali, adjusted to pH 7.8–8.0, and lyophilized. This precipitate contains the cells not sedimented by Sharples centrifugation, vari-

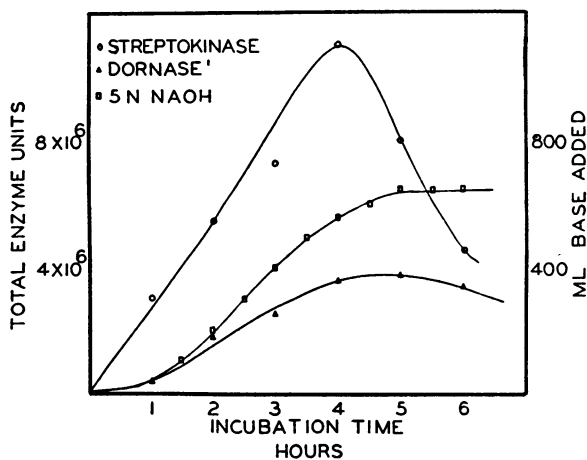


FIG. 1. RELATION OF STREPTOKINASE AND DESOXYRIBONUCLEASE PRODUCTION TO GROWTH (EXPRESSED AS ML. 5N NaOH ADDED TO NEUTRALIZE ACID FORMED)

¹ Desoxyribonuclease.

ous streptococcal proteins, some media components, nucleic acids, streptokinase and desoxyribonuclease.

2. *Purified streptokinase*: The purification procedure is a modification of an earlier method (3). A 2–5% solution of crude streptokinase is made

in borate buffer, pH 7.8–8.0. Cells and insoluble debris are spun out at 16,000 rpm in a refrigerated angle centrifuge. Occasionally a significant portion of the activity is also spun down by this treatment. If this is the case, the precipitate is resuspended and made alkaline to thymol blue (above pH 9). The solution is then adjusted to pH 7.8–8.0 and centrifuged. Following this treatment the activity remains in solution. The supernatants are pooled and diluted with distilled water to a nitrogen content of about 0.25–0.50 mg./ml. and sufficient Pillimer's acetate buffer is added (4) to reduce the pH to 5.6. Sufficient 1% protamine³ solution in distilled water is added to cause the formation of a flocculent precipitate (usually about 1 ml./5–10 mg. nitrogen). The protamine precipitate is spun out in a refrigerated angle centrifuge at 4,000–5,000 rpm. The supernatant should show no further precipitation on testing with protamine, but care should be used to avoid any great excess of protamine, since it will complicate later purification steps. The supernatant is cooled to about 0° and ethanol at –10° to –20° is added to a final concentration of 20%. On standing overnight at –8° to –10° a flocculent precipitate forms which is spun off in an angle centrifuge in the cold. The precipitate is dissolved in borate buffer, pH 7.8–8.0. Some insoluble material remains in suspension, but in most cases little activity is associated with it; and it can be removed by centrifugation, resulting in little or no decrease in total activity, but a decrease of about 50% in total nitrogen. The clear solution usually contains between 100 and 180 units of streptokinase per microgram of nitrogen. Recovery usually represents about 25% of the original activity of the culture, some 50% of the original activity being lost during the initial Sharples centrifugation. Desoxyribonuclease, which has accompanied streptokinase in the previous purification steps, is separated from streptokinase by the 20% ethanol fractionation.

³ Several sources of protamine have been tested. In our experience, consistently good results are obtained with Squibb protamine. Protamines from other sources often fail to effect purification, and are less efficient in that much larger quantities are necessary to precipitate inert materials. We wish to express our gratitude to E. R. Squibb and Sons for generous gifts of protamine.

Practically all of the desoxyribonuclease activity remains in the supernatant.

The purified streptokinase is sterilized by filtration through Seitz or Sela filters, lyophilized and stored in the refrigerator. Solutions remain stable for several weeks if not too dilute and if kept in the cold.

3. *Streptococcal desoxyribonuclease*: As mentioned in previous reports (5–7), streptococcal cultures contain a potent desoxyribonuclease. In the original method of purification (2) using ammonium sulfate, both enzymes were found in the same fraction. However, they are readily separated since streptokinase is precipitated at pH 5.6 by 20% ethanol or 50% ammonium sulfate, while the majority of the desoxyribonuclease remains in solution. In the present study, desoxyribonuclease was prepared by bringing the 20% ethanol supernatant of the above protamine-purified streptokinase precipitate to 40–50% ethanol. The precipitate which forms overnight in the cold is collected by centrifugation and dissolved in the gelatin-magnesium-veronal buffer recommended for desoxyribonuclease titration by McCarty (8). The preparations usually contain insoluble material, with which is associated some of the desoxyribonuclease activity. The clear supernatant obtained by centrifugation of the material, however, contains a significant proportion of activity.

Up to the present time methods have not been perfected for the quantitative isolation of the enzyme from streptococcal cultures and recovery represents only a fraction of the amount in the

TABLE I
Fractionation of streptokinase and desoxyribonuclease from streptococcal cultures

Fraction	Total units	
	Streptokinase	Desoxyribo-nuclease
(1) original culture	1.75×10^6 units	5.25×10^4 units
(2) after Sharples centrifugation	1.5×10^6	4.1×10^4
(3) alcohol precipitate of culture supernatant	$1.2 \times 10^{6*}$	3.0×10^4
(4) 20% alcohol ppt. of protamine supernatant	1.38×10^6	0.1×10^4
(5) 40% alcohol ppt. of (4) supernatant	0.01×10^6	1.8×10^4

* This value is somewhat lower than the true value because sufficient time for reversal of the inactivation, which occurs in the neighborhood of pH 5 (3), was not allowed before titration.

original culture. This loss is due apparently to the instability of the enzyme.

In Table I are presented the data obtained in a typical purification experiment.

4. *Bovine fibrinogen*: For reasons to be discussed later, human fibrinogen preparations were considered unsuitable for assay purposes. Armour's Fraction I of bovine plasma,⁴ a commercial product, has been found to be quite suitable for our purposes, since it contains no antistreptokinase, serum inhibitor or plasminogen activatable by streptokinase. The dry powder is stored in the refrigerator and dissolved in borate buffer, pH 7.4-7.6, in appropriate concentrations before use. Solutions are reasonably stable and generally remain usable for six to eight hours if kept cool.

5. *Plasminogen*: No satisfactory preparation of plasminogen is available. The purer preparations with which we are familiar, such as Howard Fraction III-2,3 (9), contain plasmin as well as plasminogen and proteolytic products of fibrin. The cruder preparations, prepared by salt fractionation (2), isoelectric precipitation (2, 10) or ethanol fractionation, such as Harvard Fraction III (11), contain a variety of substances, some of which undoubtedly influence the streptokinase-plasminogen reaction. However, reproducible results have been obtained in these laboratories by using a single lot of Harvard Fraction III.⁵ For use, the material is made up in borate buffer in a concentration of 0.25%. The solutions must be used immediately, and kept in an ice bath, because some spontaneous plasmin activity will develop in a few hours. However, when used as outlined, we have noted no variation in the plasminogen titer of the dry material in over a year.

6. *Standard antistreptokinase*: A pool of Harvard Fraction II-III was fractionated by Harvard method 9 (12). Fraction II, the antibody fraction, was lyophilized and stored in a vacuum dessicator.⁶ Assay of the material with strepto-

kinase indicated that 1 mg. would inhibit the activity of 10 units of streptokinase, when tested by the method outlined below. The material was assigned, therefore, a value of 10 antistreptokinase units per mg., and used throughout these studies as a primary standard to check the constancy of the streptokinase unit and of the antistreptokinase titrations.

7. *Crystalline trypsin*: A solution of commercial crystalline trypsin, obtained from the Plaut laboratories, is prepared in N/400 HCl. The stock solution contains 0.1% of the dry powder, of which about 50% is magnesium sulfate. The concentration of active trypsin in the solution is determined by titration with soybean inhibitor, as outlined below, and appropriate dilutions for use, usually containing 20 μ g. of active trypsin per milliliter, are made up in borate buffer immediately before use.

8. *Crystalline soybean inhibitor*: A stock solution of Kunitz' crystalline inhibitor⁷ (13), containing 2 mg./ml. of inhibitor, was prepared in borate buffer. Appropriate dilutions are made up in borate buffer, pH 7.4-7.6, before use.

9. The preparation of other reagents, such as borate buffer and gelatin buffer, have been detailed in previous reports (2, 14).

METHODS

1. *Streptokinase assay*: Many methods have been described for streptokinase assay, utilizing clots prepared from human fibrinogen (15-18). These methods depend on the presence of plasminogen as a contaminant of the fibrinogen preparations, since, as Milstone (10) has shown, highly purified human fibrinogen will not lyse in the presence of streptokinase unless another serum factor (lysin factor, plasminogen) is added. In our experience, methods utilizing human fibrinogen are unsuitable for long-term routine use. The principal disadvantage is the variation from batch to batch in the fibrinogen-plasminogen ratio obtained with different methods of prepa-

⁴ We are indebted to Dr. J. B. Lesh of Armour Laboratories for supplying the Fraction I used throughout this study. Fraction I is an ethanol fraction of bovine plasma and consists largely of fibrinogen.

⁵ We are indebted to Dr. Dwight Mulford, Division of Biologic Laboratories, Massachusetts State Department of Health, for supplying a large lot of Fraction III isolated from a single pool of plasma.

⁶ The author wishes to express his indebtedness to Dr. J. W. Williams and associates of the Department of

Physical Chemistry of the University of Wisconsin for making facilities available and for help in the preparation of various plasma fractions.

⁷ Dr. M. Kunitz kindly supplied us with a preparation of crystalline soybean inhibitor for use as a standard, and made available his assay methods in advance of publication.

ration, and even with the same method. For example, in our experience ethanol fractions of plasma, such as Harvard I, contain less plasminogen than do the salt fractionations previously used in this laboratory (2), as shown by the fact that clots treated with streptokinase will lyse much more readily if the fibrinogen is prepared by salt fractionation rather than the ethanol. Also, human fibrinogen preparations are unstable, due to the spontaneous conversion of plasminogen to plasmin. Another difficulty in the use of human fibrinogen is the presence of variable amounts of antibody globulin in the preparations. While on these occasions no particular difficulty with most samples of normal plasma whatever the method of fractionation, we have encountered occasional lots of plasma with such high titers of antistreptokinase that they were unusable.

For these reasons, we have modified the test by the substitution of clots prepared from bovine fibrinogen (Armour's Fraction I). The bovine fibrinogen contains no plasminogen or plasmin, as shown by the fact that such clots, without added protease, will remain stable for days in the presence or absence of streptokinase. Antistreptokinase and inhibitor titrations indicate the absence of these substances also. As a source of plasminogen we have used a single large lot of Harvard Fraction III. The fibrinogen concentration used is 0.1%, as in previous reports, and the concentration of Fraction III is adjusted to give a streptokinase unit equal in value to the one previously employed in this laboratory (3). The streptokinase assay is performed as follows:

- 0.1 ml. streptokinase dilution in gelatin buffer
- 0.4 ml. bovine fibrinogen, 0.25% in borate buffer
- 0.5 ml. Fraction III, 0.25% in borate buffer.
(This solution must be prepared just before use, and should be kept on ice)
- 0.1 ml. Lederle Hemostatic Globulin,^{*} diluted 1:3 in borate buffer.

^{*} Lederle Hemostatic Globulin has recently become unavailable. Parke-Davis bovine thrombin may be substituted as well as Upjohn thrombin. They are less stable than Hemostatic Globulin when in solution in borate buffer, and solutions must be titrated frequently to determine the minimum amount necessary to form a firm clot. These preparations are more stable in glycerol solution, but we have not used glycerol solutions.

The tubes are incubated at 35° and the lysis time of each tube of the series noted. The dilution lysing the clot in 10 minutes contains one unit and is determined by interpolation as outlined in a previous report (3).

2. Antistreptokinase: The antistreptokinase unit is defined as the amount of antibody required to inhibit 1 unit of streptokinase to such a degree that it will not activate the plasminogen sufficiently to cause lysis of the standard test clot in 30 minutes. Other methods of antistreptokinase assay have been presented (15, 18-20) but no general agreement has been reached on a method. We have thought it preferable to base the antibody unit on the method of streptokinase titration used in this laboratory. In order to have a primary standard of reference throughout the study, a lot of Fraction II, the gamma globulin fraction of human serum, was prepared and lyophilized. This preparation contained 10 antistreptokinase units per mg., and has been used as a reference standard for streptokinase and antistreptokinase titrations. Standardization of streptokinase with standard antibody is performed as follows:

- 0.5 ml. streptokinase dilutions in gelatin buffer, pH 7.6-7.8
- 0.5 ml. standard antibody solution, containing 2 antistreptokinase units per ml. (The antibody solution is heated at 56° for 30-45 minutes to destroy the small amount of plasminogen and inhibitor which it contains.)

The mixture of streptokinase and antibody is incubated at 35° for 30 minutes. At the end of this time, the following reagents are added:

- 0.5 ml. of a 0.25% solution of Fraction III
- 0.5 ml. of 0.5% bovine fibrinogen
- 0.1 ml. Hemostatic Globulin, diluted 1:3.

The tubes are incubated for another 30 minutes and the highest dilution of streptokinase which fails to lyse the clot is taken as the end point. A solution of crude or purified streptokinase standardized in this manner should show no significant change in titer over a period of a week or two if the streptokinase concentration is above about 2,000 units per ml. for the crude and above

10,000 units per ml. for the purified. More dilute solutions are not quite so stable, probably because of denaturation occurring in dilute protein solutions. However, in practice, the streptokinase solution is standardized every day or two.

In determining the antistreptokinase titer of serum the serum must be heated at 56° for 30–45 minutes to destroy the plasminogen and inhibitor which it contains. As may be seen in Table II,

TABLE II
Effect of heating serum at 56° C for 30 minutes on serum inhibitor and antistreptokinase

Serum number	Inhibitor equivalent to micrograms trypsin		Antistreptokinase	
	before heating	after heating	before heating	after heating
1	1000	100	128	128
2	500	80	64	64
3	1000	—80*	64	32
4	667	100	64	32
5	667	—80*	128	64
6	667	—80*	16	8
7	1800	120	2560	2560
8	800	48	3200	3200

* Inhibitor equivalent to less than 80 µg.

heating reduces the inhibitor concentration by approximately 90%, and produces no significant change in the antibody titer. A more valid reason for heating the serum than for the inactivation of inhibitor is to destroy plasminogen, since if this is not done, in the case of sera with a low antibody content, the tubes containing the lowest dilutions of serum will lyse the standard clot because of the activation of the plasminogen in the serum by uncombined streptokinase.

The inactivated serum is titrated for antistreptokinase as follows:

0.5 ml. inactivated serum dilution

0.5 ml. streptokinase solution, diluted in gelatin buffer to contain about 1–2 units.

The tubes are incubated for 30 minutes, and the remainder of the titration carried out as above. The end point is taken as the highest dilution of serum preventing lysis of the clot, and contains antistreptokinase equivalent to the streptokinase in the test.

3. *Serum inhibitor*: A number of methods have been devised in the last few years for the quantitative estimation of serum inhibitor (14, 18, 21–

23). These methods, however, have certain disadvantages for routine use.

Kunitz (13) has shown that crystalline soybean inhibitor undergoes an immediate, stoichiometric, irreversible reaction with trypsin, and that active trypsin can readily be determined by titration of the amount of soybean inhibitor necessary to inhibit the proteolytic activity of the trypsin sample, since the two are equivalent, weight for weight. Schmitz (24) has isolated a small amount of inhibitor from serum which he concluded was probably identical with the pancreatic inhibitor. It has also been shown (14) that serum and pancreatic inhibitor behave in the same way toward trypsin and toward plasmin, although the two inhibitors behave toward trypsin in a quantitatively and perhaps qualitatively different manner than they behave toward plasmin. In view of these facts it was decided to determine the inhibitory power of serum in terms of its ability to inhibit trypsin rather than plasmin. Additional reasons influencing this decision were as follows: (a) crystalline trypsin is readily available and easily standardized, whereas no plasmin preparations of comparable purity are known; (b) trypsin is much more susceptible to the action of inhibitor than is plasmin; and (c) active trypsin can be conveniently stored in a stable solution (in M/400 HCl) while plasminogen and plasmin preparations are very unstable (14, 25, 26).

Standardization of trypsin with soybean inhibitor is carried out as follows:

0.5 ml. freshly prepared dilutions of stock trypsin in borate buffer, pH 7.4–7.6

0.5 ml. soybean inhibitor, usually containing 10 µg.

1.0 ml. bovine fibrinogen, 0.2% in borate buffer.

The tubes are incubated for 10 minutes at 35°. At the end of this time 0.1 ml. of Hemostatic Globulin, diluted 1:3, is added and the formation of clots noted in 5–10 minutes. The method is based on Ferguson's (27) procedure for the fibrinogenolytic assay of tryptases. The lowest dilution of trypsin in which a clot forms contains trypsin equivalent to the soybean inhibitor in the test. It is necessary to allow the trypsin to act in the absence of Hemostatic Globulin because the Hemostatic Globulin preparations used con-

tain some trypsin inhibitor.⁹ An advantage to this procedure, however, is that the addition of Hemostatic Globulin stops all tryptic activity, so that once the clots are formed, they are stable. Any desired range of trypsin concentration can be titrated by this method by using appropriate concentrations of inhibitor in the test. The sensitivity obtained depends on the interval between successive trypsin dilutions.

The inhibitor concentration of serum is determined as above with the substitution of dilutions of serum for the soybean inhibitor and the substitution of a fixed amount of active trypsin, usually about 10 μ g., for the trypsin dilutions. The tube containing the highest dilution of serum in which a clot forms on addition of thrombin contains serum inhibitor equivalent to the trypsin in the test. Results are expressed as micrograms of active trypsin inhibited by 1 ml. serum.

The accuracy of the fibrinogenolytic test was compared with the method of Kunitz (13) for determining trypsin inhibitor, with results as shown in Table III.

TABLE III
Comparison of methods for serum inhibitor determination
Inhibitor equivalent to micrograms trypsin
inhibited per ml. serum

Serum number	Kunitz method	Fibrinogenolytic method
1	520	500
2	490	500
3	590	660

4. *Plasminogen*: Plasminogen determinations of serum are of doubtful accuracy because plasminogen cannot be separated quantitatively from serum, nor can the interfering substances, antistreptokinase and serum inhibitor, be removed or destroyed without loss of plasminogen. However, in samples of sera which are not abnormally high in antibody or inhibitor, the relative concentrations of plasminogen can be determined by activating with streptokinase and determining the least amount of activated serum necessary to lyse a standard clot. The details of the determination are as follows:

0.5 ml. serial dilutions of serum in borate buffer

⁹ As noted above, Hemostatic Globulin is no longer available. Preliminary experiments with the more highly-purified bovine preparation of Parke-Davis indicates that they contain little or no inhibitor.

0.1 ml. streptokinase solution, previously standardized as outlined below

0.4 ml. bovine fibrinogen, 0.5% in borate buffer
0.1 ml. Hemostatic Globulin, diluted 1:3.

The highest dilution of serum lysing the clot in 30 minutes at 35° represents one unit of plasmin, or plasminogen.

It is necessary to activate the plasminogen in the presence of substrate, because, if this is not done, the total protease activity declines rapidly, probably because of the two-fold effect of auto-digestion (25) and combination with serum inhibitor. Activation in the presence of substrate appears to minimize these effects. Since the activation of plasminogen by streptokinase appears to be a catalytic reaction (2, 14, 28-31) it will be most efficient when an excess of streptokinase is used. A large excess of streptokinase would also prevent interference with the reaction by the antistreptokinase contained in variable amounts in practically all human sera. When attempts are made to use maximal amounts of crude streptokinase to activate serum plasminogen, an inhibitory effect is noted. This inhibitory effect of undiluted, concentrated crude streptokinase is due, probably, to the presence in crude streptokinase preparations of a protease inhibitor (14) which decreases in amount as streptokinase is purified. When purified streptokinase is used to activate a serum, maximum protease activity is obtained with the highest concentration of streptokinase, as shown in Table IV.

TABLE IV
Plasminogen activity of serum activated with various concentrations of purified streptokinase

Purified streptokinase dilution	Highest dilution of serum causing lysis in 30 minutes
undiluted	5120
1:10	5120
1:100	1280
1:1000	160

In addition to the absence of an inhibitory effect of high streptokinase concentration when using purified material, it can be seen from the data in Table V that the activity obtained is considerably higher than with the crude material. It is obvious, therefore, that activation with purified material is the method of choice. Unfortunately, it has not been possible in the past to obtain sufficient purified material for all requirements of the stud-

ies involved in these reports. Therefore, protease determinations were made with selected batches of crude streptokinase, titrated to determine those lots and dilutions which would give the highest

are presented the results obtained by using the methods described above.

TABLE V
Relation between maximum plasmin activity developed in serum by crude and purified streptokinase

Serum	Maximum plasmin activity	
	Crude streptokinase	Purified streptokinase
L (6-19-48)	1280	3200
L (7-16-48)	640	1600
S	320	1280
B	160	1280
F	320	2560

values with normal sera. Such lots were set aside and used in all the protease determinations reported in the present studies. The results obtained, while not representing the maximal values, are useful as a measure of the relative plasminogen content of sera. The validity of this assumption is borne out by the observation (Table V) that a direct relation exists between the maximum amount of protease activity obtained on the activation of serum with crude and with purified streptokinase.

5. *Desoxyribonuclease*: The determination is carried out as described by McCarty (7), except that drop in relative viscosity is measured over a 10-minute instead of a 20-minute period of incubation. A unit is defined as the amount of enzyme necessary to cause a drop of 1 viscosity unit in 10 minutes at 30° in a reaction mixture consisting of 2.4 ml. of desoxyribose nucleic acid and 0.1 ml. enzyme solution. The substrate consisted of a 0.15-0.2% solution of thymus desoxyribose nucleic acid in veronal buffer. The nucleic acid was prepared as described by Mirsky (32). Difficulty was encountered with the nucleic acid solutions in that a spontaneous drop in viscosity was noted. This effect could be eliminated by heating the nucleic acid solution to 56° for about an hour. The heated solution, when used in the reaction mixture described above, had an initial relative viscosity of 4-5 units.

RESULTS

1. *Antistreptokinase*: Evidence is abundant that an antibody to streptokinase appears following streptococcal infection (18, 33-36). In Table VI

TABLE VI
Antistreptokinase levels in various sera

Serum	No. samples	Antistreptokinase units	
		Mean	Range
Normal	14	36	4-80
Infants (under 1 year)	9	8	0-29
Rheumatic fever (children)	8	1302	100-4200
Streptococcal pharyngitis	3	92	80-115
Tuberculous pleurisy	22	87	2-400
Dog	3	102	72-156
Monkey	1	66	
Cow (pool)		8	
Horse (pool)		4	
Chicken	1	32	

Direct evidence that antistreptokinase is produced in humans following injection of streptokinase has been obtained in the present series of studies and is reported more fully in the accompanying paper (1). In Table VII are shown the antibody titrations in the serum of two patients following the intrapleural injection of purified streptokinase. The antibody responses seen in the two patients reported in Table VII are perhaps

TABLE VII
Rise in antistreptokinase following intrapleural injection of purified streptokinase

Patient	Date	ASK units
M. H. (female)	6/8 (200,000 SK injected intrapleurally)	20
	6/11	80
	6/17	5120
	6/22	over 10,000
C. L. (male)	5/13 (50,000 units Sk injected intrapleurally)	40
	5/17 (100,000 units SK)	—
	5/20	5120
	6/9	over 10,000
	6/22	over 10,000
	7/2	5120

somewhat more rapid than is usual as a primary response to the injection of an antigen. The rapidity of the response, however, is probably due to the fact that the response is an anamnestic one, since it will be noted that both patients had a titer before injection of streptokinase. In fact, all human sera tested have had some antibody, even

with no known history of streptococcal infection.

2. *Serum inhibitor*: The values obtained for a variety of sera are presented in Table VIII. Results are expressed as micrograms of active trypsin inhibited by 1 ml. of serum.

TABLE VIII
Serum inhibitor levels of serum from man and animals

Serum	No. of samples	Serum inhibitor as micrograms trypsin inhibited per ml.	
		Mean	Range
Normal adult	16	655	100-1200
Infants (under 1)	9	613	420-1024
Rheumatic children	5	760	570-900
Pulmonary tuberculosis	7	1035	660-1500
Carcinoma	4	883	600-1333
Leukemia	5	1096	842-1350
Chicken	1	264	
Rabbit	1	400	
Horse (pool)	2	765	670-860
Dog	1	365	
Guinea pig	2	2125	1750-2500

The data presented are obviously too scanty to warrant the drawing of conclusions. However, the finding that the value is elevated in chronic wasting diseases such as tuberculosis and malignancies is in accord with many earlier observations on serum inhibitor (see Grob [21] for references).

The values for animal sera tested lie within the range of normal human values, with the exception of guinea pigs, which, at least in the few specimens tested, have an abnormally high value.

TABLE IX
Levels of plasminogen in various sera

Serum	No. of specimens	Units of plasminogen	
		Mean	Range
Normal adult	8	572	160-1280
Rheumatic children	3	960	320-1280
Tuberculosis	6	4053	1280-5120
Carcinoma	1	2560	
Non-rheum. H. D.	5	576	320-640
Chicken	1	Less than 10 units	
Monkey	2	Less than 10 units	
Dog	3	Less than 10 units	
Cattle (pool)	1	Less than 10 units	

Of interest are the somewhat higher values for rheumatic children. If these differences are significant, their relation to the higher plasminogen values obtained for rheumatics (Table IX) is of

importance, suggesting the possibility that the inhibitor might serve a role in relation to plasminogen. However, when one considers the inhibitor and plasminogen values obtained with animal sera, no relationship appears to exist, since the inhibitor level is high while the amount of plasminogen activatable by streptokinase is negligible.

3. *Plasminogen*: The deficiencies of the method for plasminogen determination have been discussed above. The results obtained are presented in Table IX.

In spite of the deficiencies of the method, the results obtained with normal sera have been reasonably uniform, the great majority of them showing levels of 320 or 640 units. Retitration of a single serum, or titrations of successive bleedings of the same individual show a variation of no more than one tube in the series. The higher values obtained with certain of the patients under investigation (*e.g.*, tuberculous and carcinomatous) probably represent an increase over normal, even though the method of titration does not give maximal values for any serum. If these values are artifacts and do not represent an increase of total plasminogen over normal, it is difficult to account for the results, since the two substances in serum known to influence the reaction, serum inhibitor and antistreptokinase, would both tend to decrease, rather than increase the plasminogen values.

SUMMARY

A method for purification of streptokinase describes conditions for obtaining high yields from bacterial cultures. Protamine is used to remove impurities and streptokinase is separated from desoxyribonuclease with cold ethanol at 20% concentration. The desoxyribonuclease is precipitated with 40-50% cold ethanol. The yield of streptokinase is about 25% and the product so obtained has been used in clinical studies.

Methods are briefly described for the quantitative estimation of streptokinase, antistreptokinase, serum inhibitor, serum plasminogen, and desoxyribonuclease. These methods were employed to make a survey study of various sera. There is a marked rise in antistreptokinase activity following intrapleural injection of streptokinase in human beings. Serum from the normal

adult contains more antistreptokinase than serum from the infant and the level is strikingly high in rheumatic fever. In various species the concentration decreases in the following order: dog, monkey, man, chicken, cow and horse. Serum inhibitor levels show elevations in wasting diseases, and in the animals studied the guinea pig has an exceptionally high inhibitor titer. The method for plasminogen determination presents difficulties but it appears that there may be a marked increase in patients with tuberculosis. The concentration in chicken, monkey, dog, and cattle appears to be low.

Serum can be heated at 56° C for 30 minutes to destroy serum inhibitor but antistreptokinase is not destroyed.

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