PROTAMINE PURIFICATION OF STREPTOKINASE AND EFFECT OF pH AND TEMPERATURE ON REVERSIBLE INACTIVATION*

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INTRODUCTION

In the course of investigations on the purification and properties of streptokinase, it was noted that a marked loss of activity occurs over a fairly narrow acid pH range (Christensen, 1940), and more recently, that the loss can be completely reversed when solutions inactivated in this way are made slightly alkaline. The phenomenon occurs with crude concentrates as well as with partially purified preparations of streptokinase. In the present report, methods for the preparation of more highly purified streptokinase are described, together with the effect of pH and temperature on reversible inactivation.

Materials and Methods

1. Streptokinase.—The preparation of crude streptokinase concentrates has been described in a previous report (Christensen, 1945). These concentrates have been pooled and stored as precipitates under saturated ammonium sulfate in the refrigerator. For use in the present experiments, aliquots of this pool were removed, diluted with an equal volume of 1 per cent acetic acid, and the precipitates spun out in an angle centrifuge. The precipitates were then washed with 1 per cent acetic acid until sulfate-free, and dissolved in saline buffer with the aid of alkali, or in 0.05 N NaOH in the cold. It is necessary to suspend the precipitates in an alkaline medium, otherwise a large proportion of the active material will not dissolve. As will be shown in the present paper, streptokinase undergoes irreversible inactivation above pH 9. However, if the material is exposed to alkali for only brief periods in the cold, this inactivation is negligible. Insoluble debris was spun out in an angle centrifuge in the cold, the supernatant adjusted to pH 7.5–8.0, and the final protein concentration to 1 to 2 per cent. Solutions prepared in this way are fairly stable when kept in the refrigerator, and have been used as starting material in the purification procedure to be described below as well as in certain of the inactivation experiments, where they are referred to as "crude concentrates."

2. Protamine.—A 1 per cent solution of Squibb protamine in distilled water was used.

3. Fibrinogen.—Lyophilized human plasma[1] was dissolved in 0.1 per cent citric

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[1] The dried human plasma was made available by the Blood Donor Service of the American Red Cross.

acid. The fibrinogen and some of the globulins were precipitated by bringing the solution to ⅓ saturation by the addition of ½ volume saturated ammonium sulfate solution. The precipitate was removed by centrifugation and washed once or twice with ⅓ saturated ammonium sulfate solution, dissolved in the minimal amount of saline buffer at pH 7.2–7.5, and lyophilized.

4. Thrombin.—The Lederle preparation, "hemostatic globulin," diluted 1:5 with saline buffer, was used.

5. Buffers.—The saline and gelatin buffers were the same as those employed in previous experiments (Christensen and MacLeod, 1945). The pH was 7.4–7.5, unless otherwise noted.

6. Streptokinase Assay.—This method is a modification of procedures used by a number of investigators (Van Deventer, 1934–35; Fraser and Madison, 1935; Madison and Taranik, 1937; Christensen, 1941; Kaplan *et al.*, 1946) involving the determination of the smallest amount of streptokinase that will cause lysis of a standard fibrin clot in a given time. 0.1 ml. of serial dilutions of streptokinase in gelatin buffer, 0.8 ml. of standard fibrinogen solution, and 0.1 ml. of thrombin solution are mixed and placed in a water bath at 35°C. The fibrinogen clots in 1 minute or less. The lysis time of the tubes of the series is followed for about 20 minutes, and the lysis time of each dilution of streptokinase is plotted against the reciprocal of the dilution on log-log paper. A straight line can be drawn through the points, beginning with a lysis time of 1½ to 2 minutes and extending to about 20 to 30 minutes. Above and below these points the curves cannot be used for purposes of calculation. The minimal lysis time of fibrin clots prepared as described above, is about 1½ minutes, and the addition of more concentrated streptokinase preparations has not reduced the lysis time significantly below this level. Lysis times longer than 30 minutes are also not reliable, since with some batches of fibrinogen curves are obtained which deviate from the extrapolated 2 to 20 minute curve. Further, on prolonged incubation some fibrin clots have a tendency to contract into a small ball, which not only prolongs lysis, but renders determination of complete lysis more difficult. By definition, one unit of streptokinase is contained in the amount of solution which will lyse the clot in 10 minutes, as determined by interpolation on the lysis time curve.

7. Standardization of Fibrinogen.—The proper concentration of fibrinogen to be used in the test is determined in the following manner: Serial dilutions of a 1 per cent solution of the lyophilized human fibrinogen preparation are prepared in saline buffer. These dilutions are set up as in the assay method just described, with buffer substituted for the streptokinase solution. The highest dilution of fibrinogen which gives a firm clot is then titrated with a streptokinase solution of known activity. In general, it has been found that this concentration of fibrinogen will give streptokinase values within 10 per cent of those obtained with other preparations of fibrinogen similarly standardized. If the difference is greater than 10 per cent, the fibrinogen concentration should be increased or decreased slightly so that lysis time values in agreement with the previous preparations are obtained. Obviously, this method is subject to many inaccuracies, not the least of which is the possibility of cumulative errors in the comparison of new batches of fibrinogen with previous ones. Since some of the antibody globulins are precipitated with fibrinogen, care should be exercised to avoid the selection of plasma with a high antistreptokinase content, for example, the plasma of

patients with rheumatic fever, or convalescent from streptococcal infections. However, the method gives reproducible results when the same batch of fibrinogen is used, and in the studies to be reported below, the same batch was used throughout any one experiment.

EXPERIMENTAL

1. Protamine Purification of Streptokinase.—The behavior of crude concentrates of streptokinase suggested the presence of nucleoprotein or nucleic acid-protein complexes. Chemical analysis for ribose and phosphorus indicated a nucleic acid content of about 20 per cent. Tests for desoxyribose were negative. Electrophoretic analysis[2] of a sample at pH 8.6 in barbiturate buffer showed four major components, one of which had a high mobility similar to that of nucleic acid, and represented about 20 per cent of the total material.

Because of the difficulty in purifying proteins containing large amounts of nucleic acid, attempts were made to remove this component before proceeding with further fractionation. It was found that under the proper conditions both the nucleic acid and about 90 per cent of the other nitrogenous material were precipitated by protamine, leaving the active streptokinase in solution. Thus, by a single step, an eight- to tenfold increase in the purity of the preparation was accomplished. The details of a typical preparation are given below.

Preliminary experiments had shown that the active material does not precipitate with protamine at pH 5–6.5, while other proteins in the concentrate do. Above pH 6.5 increasing amounts of streptokinase are precipitated along with inert constituents, until at about pH 7.5 none of the active material remains in the supernatant.

500 ml. of crude concentrate was adjusted to pH 5.5–5.6 with acetic acid. 500 ml. of 1 per cent protamine was added with stirring and the precipitate spun out in an angle centrifuge. The supernatant was saturated with solid ammonium sulfate and allowed to stand in the refrigerator. The insoluble protamine precipitate was extracted several times with 1 per cent sulfuric acid to remove the protamine, and the extracts saved for recovery of the protamine. The precipitate was dissolved in dilute alkali and adjusted to pH 8. Since this solution was found to contain a large amount of streptokinase, the precipitation with protamine was repeated, and the supernatant, after saturation with ammonium sulfate, was combined with the first protamine supernatant in the refrigerator. The ammonium sulfate precipitate was collected by filtration on hard paper, dissolved in saline buffer at pH 8.0, precipitated by acidification with glacial acetic acid, and washed with dilute acetic acid until sulfate-free. The precipitate was then dissolved in saline buffer, pH 8, and lyophilized.

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[2] We are greatly indebted to Dr. Theodore Shedlovsky of The Rockefeller Institute for Medical Research for electrophoretic analysis of several streptokinase preparations.

In Table I are shown the nitrogen, phosphorus, and activity content of the crude and purified material.

Although protamine purification results in removal of nucleic acid and the majority of the inactive nitrogenous material, and increases the activity from about 16 units of streptokinase per microgram of nitrogen to about 125 units, the material is still not pure, since electrophoretic analysis reveals several components. At pH 8.6 in barbiturate buffer[2] the material did not resolve into symmetrical peaks. At pH 6.5 in cacodylate buffer[3], three distinct peaks were seen. One peak moved toward the negative pole very rapidly, and probably represented protamine incompletely removed from the preparation. The other two peaks, approximately equal in area, had a very low mobility.

In several of its properties purified streptokinase preparations differ from earlier, cruder preparations. The purified material is much more soluble at neutrality than the original concentrate, possibly due to the removal of nucleic

TABLE I

Purification of Streptokinase by Treatment with Protamine

Streptokinase preparation	Total nitrogen	Total phosphorus	Total activity	Activity per gamma of nitrogen
	gm.	*mg.*	*units*	*units*
Crude concentrate	1.95	177.5	32×10^6	16.4
Protamine purified	0.227	Less than 4	28×10^6	123.5

acid. Moreover, the protease inhibitor present in crude preparations (Christensen and MacLeod, 1945), has been largely, if not entirely removed during the protamine purification.

2. The Effects of pH and Temperature on Streptokinase.—In the experiments to be described, a single preparation of purified streptokinase was used throughout. However, similar results have been obtained with other purified preparations as well as with crude concentrates.

(*a*) *pH Inactivation.*—A sample of purified streptokinase was divided into aliquots, each of which was adjusted to a different pH value and incubated at 56°C. for 60 minutes. At the end of this time the pH of the samples was adjusted to 7.4, they were made up to constant volume in gelatin buffer, and tested immediately for residual streptokinase activity. The residual activities of the samples, as a function of pH, are plotted in Fig. 1.

From the data shown in Fig. 1 it can be seen that at a pH of about 5 there is a marked loss of activity. Above and below this pH value the residual activity is

[3] We wish to express our appreciation to Dr. J. W. Williams and staff of the Department of Chemistry, University of Wisconsin, for placing electrophoresis apparatus at our disposal and for assistance and advice in its operation.

greater, indicating that streptokinase is most unstable at about pH 5. With this and other preparations of streptokinase the point of maximum instability

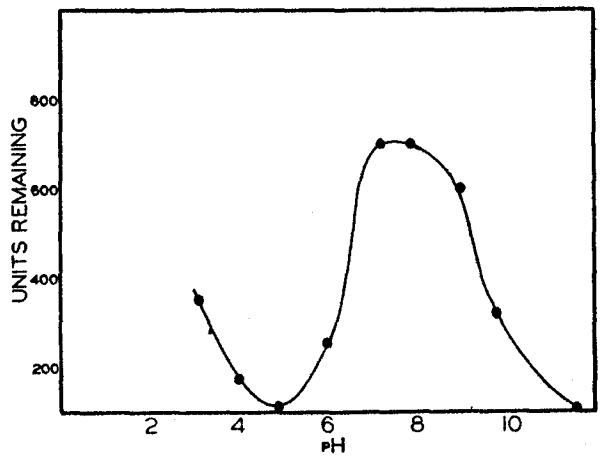

FIG. 1. Inactivation of purified streptokinase heated to 56°C. for 60 minutes a different pH values.

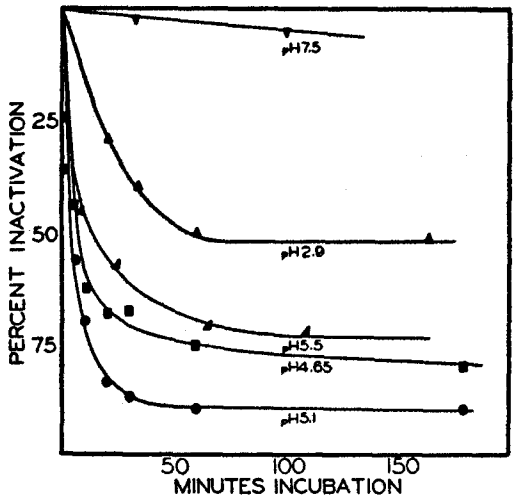

FIG. 2. Rate and extent of streptokinase inactivation at 40°C. as a function of hydrogen ion concentration.

has always fallen between pH 4.9 and 5.1. Above pH 9, streptokinase is also unstable, but the inactivation which occurs in the alkaline range is not reversible under any conditions so far employed, and has not been investigated further.

(*b*) *Rate of Inactivation as a Function of pH.*—Solutions of purified streptokinase were inactivated at various pH levels as in the previous experiment. At intervals during the inactivation, aliquots of each solution were removed and tested for residual activity. The data obtained are plotted in Fig. 2.

From the curves shown in Fig. 2 it can be seen that the rate of inactivation is a function of the pH, being greatest at about pH 5. It can also be seen that the inactivation process levels off after a time until there is very little further decrease in activity with time, suggesting the establishment of an equilibrium between active and inactive streptokinase. It appears that this equilibrium value is a function of pH, since the equilibrium value is lowest at pH 5.

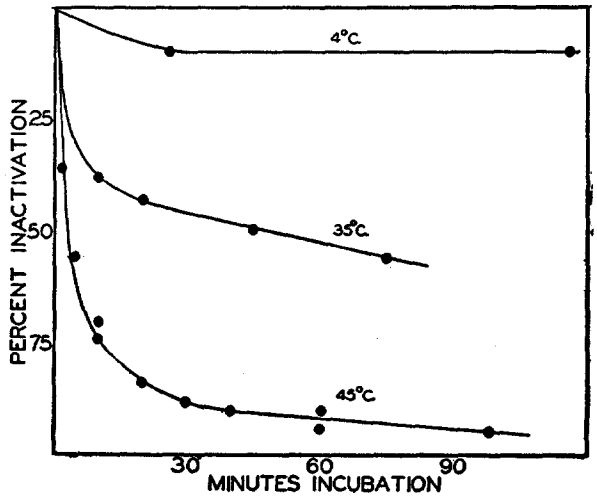

FIG. 3. Rate and extent of streptokinase inactivation at pH 5 as a function of temperature.

(*c*) *The Effect of Temperature on Inactivation.*—Solutions of purified streptokinase were adjusted to pH 5 and incubated at various temperatures. At intervals, aliquots were removed and tested for residual activity, with the results shown in Fig. 3.

The data shown in Fig. 3 indicate that the rate and extent of inactivation at pH 5 are direct functions of the temperature of incubation, the rate increasing as the temperature is raised. The dependence of the inactivation on temperature is apparently not due to a shift of the pH of maximum inactivation with temperature, since numerous experiments have shown that inactivation is maximal at pH 5 at temperatures between 25° and 56°C.

From the above data it may be concluded that the inactivation of streptokinase is dependent on both the hydrogen ion concentration and the temperature,

the rate and extent of inactivation being maximal at pH 5, and increasing as the temperature rises.

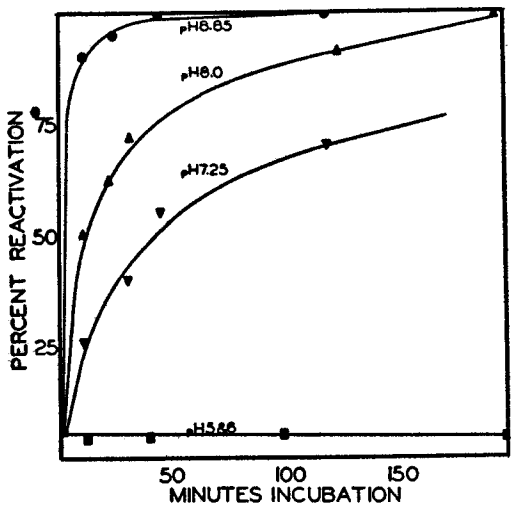

FIG. 4. Reactivation of streptokinase at 23°C. as a function of hydrogen ion concentration.

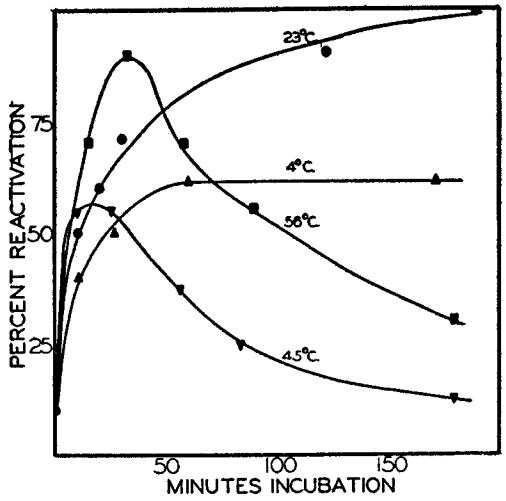

FIG. 5. Reactivation of streptokinase at pH 8 as a function of temperature.

(*d*) *The Effect of pH on Reactivation.*—Aliquots of the purified streptokinase solution, inactivated at pH 5 as in the previous experiments, were adjusted to various pH levels, made up to constant volume, and allowed to reactivate at

room temperature. At intervals, the various solutions were tested for activity, with the results shown in Fig. 4.

The data presented in Fig. 4 show that the rate of reactivation increases with increasing alkalinity. The initial rate of reactivation appears to be highest above pH 9, but a secondary inactivation occurs, and as mentioned above, this alkaline inactivation differs from the pH 5 inactivation in that it does not appear to be reversible.

(*e*) *The Effect of Temperature on Reactivation.*—A solution of pH 5-inactivated streptokinase was adjusted to pH 8.0. Aliquots were removed, incubated at various temperatures, and at intervals tested for activity. The reactivation curves obtained are presented in Fig. 5.

It can be seen from the data of Fig. 5 that the reactivation rate is a direct function of the temperature. At temperatures above 23°C., however, a secondary inactivation of streptokinase occurs, since the reactivation reaches a peak and then declines. Reversal of this secondary inactivation has not been observed.

DISCUSSION

Precipitation of the majority of the inactive protein material from culture supernatants, without precipitation of streptokinase activity, by protamine under the conditions employed suggests that streptokinase differs significantly from other proteins in the supernatants, possibly in the possession of a somewhat higher isoelectric point.

The present experiments on inactivation confirm and extend the earlier observation (Christensen, 1940) that streptokinase is unstable in the neighborhood of pH 5. The mechanism of the reversible pH inactivation is at present obscure. The data obtained in the present experiments are not adequate for an analysis of the kinetics of the phenomenon. The experiments do indicate, however, that under appropriate conditions of temperature and pH, an equilibrium is established between active and inactive streptokinase, the particular equilibrium value attained being dependent on both pH and temperature. Two possibilities may be suggested to explain this effect. Streptokinase may undergo a pH and temperature–governed interaction with other components of the partially purified material. With present preparations this cannot be ruled out. On the other hand, it is possible that the effect is due to a pH and temperature–governed alteration in the state of the streptokinase molecule, for example, some type of isomerism.

The reversible inactivation of preparations of streptokinase must be considered in studies with this material. For example, in determining antistreptokinase it is possible that error may be introduced if inactive streptokinase is present, since it may bind a portion of the antibody.

SUMMARY

1. Treatment of crude concentrates of streptokinase with protamine results in removal of about 90 per cent of the nitrogenous material, including nucleic acid and protease inhibitor, with little or no loss of activity.

2. Streptokinase solutions undergo reversible inactivation with changes in pH. The rate and extent of inactivation are dependent on pH, being greatest over a very narrow range about pH 5. The rate and extent of inactivation are also a function of temperature, both increasing with the temperature of incubation. The rate of reactivation is a function of pH and temperature, increasing as either is raised. However, as the temperature is raised above about 23°C., or the pH above about 9, a secondary, irreversible inactivation of streptokinase occurs.

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