STREPTOCOCCAL FIBRINOLYSIS: A PROTEOLYTIC REACTION DUE TO A SERUM ENZYME ACTIVATED BY STREPTOCOCCAL FIBRINOLYSIN*

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INTRODUCTION

Tillett and Garner (1933) observed that cultures or culture filtrates of certain strains of beta hemolytic streptococci bring about rapid lysis of the fibrin clot formed on adding calcium or thrombin to human plasma. This reaction they termed "fibrinolysis" and the active agent in the cultures, "fibrinolysin." Fibrinolysin has been found by other investigators to be associated most consistently with hemolytic streptococci of the Lancefield groups A, "human" C, and G (Tillett, 1938). While not peculiar to these streptococci, it is infrequently encountered in other species of bacteria.

Tillett and Garner (1933) observed in addition, that human plasma and human fibrinogen-thrombin clots are susceptible to the action of fibrinolysin, whereas plasma clots from rabbits are resistant. Other investigators have confirmed this observation and extended the studies to the fibrins of additional animal species (Tillett, 1938). LeMar and Gunderson (1940), in an extensive study of the susceptibility of fibrins from various species have shown that human fibrin is the most susceptible and that animal fibrins are usually partially or completely resistant. Resistance is not a property of the fibrin itself, since both Tillett and Garner (1933) and Madison (1934–35) have shown that resistant fibrin clots of animals become susceptible to streptococcal fibrinolysin if the animal fibrinogen is clotted with human thrombin. Similarly, if human fibrinogen is clotted with animal thrombin, these clots are also susceptible. It appears, therefore, that the resistance of animal clots is not due to insusceptibility of the fibrin itself, but to some other factor or factors.

Milstone (1941) has shown that if human fibrinogen and thrombin are purified by reprecipitation and exhaustive washing, the clots prepared from these materials will not lyse on the addition of an active culture or culture filtrate. If, however, human serum or the water-insoluble globulins from human serum are added to the system, the clots become normally susceptible to lysis. Furthermore, addition of human

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serum or the globulin fraction to rabbit plasma clots renders them as susceptible to fibrinolysis as are human plasma clots. Milstone concluded that human serum, and particularly the water-insoluble globulin of human serum, contains a component which he called "lysin factor," which is necessary, together with fibrinolysin, in order to produce the fibrinolytic phenomenon. According to Milstone, human fibrinogen-thrombin clots are susceptible to fibrinolysis because lysin factor is present as a contaminant in both these reagents; rabbit plasma clots are resistant because they lack a suitable lysin factor.

The nature of the chemical reaction or reactions associated with fibrinolysis has not been well understood. Garner and Tillett (1934) were unable to detect significant evidence of proteolysis during fibrinolysis. Jablonowitz (1937, 1939), however, has reported slight changes in the immunological specificity and salt-precipitability of fibrin following lysis, which could be interpreted as evidence of proteolysis.

It seemed probable that fibrinolysis results from proteolysis of the fibrin molecule and that failure of earlier investigators to detect significant digestion of fibrin might be due to the relatively low activity of the culture filtrates used as a source of fibrinolysin, to a low concentration of lysin factor in the system, or to a combination of these factors. In order to test these hypotheses, streptococcal fibrinolysin from large volumes of culture filtrate was concentrated and partially purified. Using this concentrated material, together with a concentrated lysin factor preparation, a study of the mechanism of the fibrinolytic reaction was undertaken in an effort to determine whether or not proteolysis of the substrate occurs, and also to investigate the nature of the interaction between fibrinolysin and lysin factor. The data which have been obtained indicate that fibrinolysis is incident to proteolysis of the fibrin molecule, and that proteins other than fibrin or fibrinogen can be digested. The data also indicate that lysin factor represents a proteolytic enzyme normally present in human serum, but in an inactive state. On the addition of streptococcal fibrinolysin, lysin factor is activated in a manner analogous to the activation of trypsinogen by enterokinase.

Materials and Methods

1. Choice of Streptococcal Strain for Fibrinolysin Production.—A "human" group C strain of *Streptococcus hemolyticus* (H46A) was used for the production of fibrinolysin. This choice was influenced by the following factors: (*a*) of over 100 fibrinolytic strains tested, strain H46A produced the most active fibrinolytic filtrates, (*b*) this strain of group C *Streptococcus hemolyticus* does not produce scarlatinal (erythrogenic) toxin, and (*c*) strain H46A is not so fastidious in its growth requirements as the majority of group A strains, so that a defined medium can be prepared more easily.

2. Medium for Production of Fibrinolysin.—The obvious advantages of a protein-free medium of defined composition in attempts to isolate a metabolic product led to the adoption of the medium which Bernheimer, Gillman, Hottle, and Pappenheimer (1942) prepared for the cultivation of strain C203 of *Streptococcus hemolyticus* group

A. The principal modification made possible by the use of strain H46A was a 75 per cent reduction in the amount of glutamine added. Unfortunately, only certain lots of casein hydrolysate supported massive growth of the strain H46A. Investigation indicated that these lots of casein contained some factor necessary for massive growth, which was lacking in most lots of casein available. Because of this difficulty, Difco neopeptone was substituted for the acid hydrolysate of casein, since massive growth could be obtained uniformly under these conditions.

The following is the composition of the medium as used throughout most of the present studies:

Neopeptone base:	Neopeptone (Difco)	150.00 gm.
	KH_2PO_4	45.00 gm.
	Cystine in HCl	1.66 gm.
	Phenol red in alcohol	50.00 mg.
	Distilled water	2500.00 ml.

Adjust the mixture to pH 7.6 with 5N NaOH and heat to boiling. Filter through paper and autoclave at 121°C. for 20 minutes.

Eleven liters of distilled water in a 20 liter Pyrex carboy equipped with a mechanical stirring device are autoclaved for 1 to 2 hours and stored in the incubator until used. Immediately before inoculation, the 2½ liters of neopeptone base, together with the following reagents, are added to the carboy:

$KHCO_3$ crystals (autoclaved and dried in the incubator)	30.00 gm.
Thioglycollic acid (Eastman technical grade)	2.25 ml.
50 per cent Cerelose (technical grade dextrose)	25.00 ml.
Addition mixture	150.00 ml.

The addition mixture has the following composition:

Uracil	150.0 mg.
Adenine sulfate	150.0 mg.
Biotin	15.0 micrograms
Nicotinic acid	15.0 mg.
Pyridoxine	22.0 mg.
Tryptophane	300.0 mg.
Calcium pantothenate	75.0 mg.
Thiamin chloride	37.0 mg.
Riboflavin	7.5 mg.
Glutamine	750.0 mg.
Salt mixture	30.0 ml.
Water to	150.0 ml.

The salt mixture is the one described by Bernheimer, Gillman, Hottle, and Pappenheimer (1942).

The carboy is inoculated with the contents of a lyophilized vial of strain H46A representing about 2 to 5 ml. of neopeptone-meat infusion broth culture, and incubated at 37°C. The low glucose concentration permits growth to become well established overnight without excessive production of acid. The following morning, 1 liter of 50 per cent Cerelose is added and incubation continued. The culture is maintained in the neutral range by addition of 5N NaOH at intervals of 15 to 20 minutes. The growth rate usually attained requires the addition of about 10 ml. of 5N base per

liter per hour. A growth rate which requires less than 4 ml. of base per liter per hour results in a concentration of fibrinolysin too low to be worth further processing.

It has been shown previously (Mueller and Klise, 1932; Bernheimer and Pappenheimer, 1942) that provided the medium is adequate in other respects, the chief factor limiting streptococcal growth is the large amount of acid produced. If the acid is continuously neutralized, growth continues until a second inhibiting factor comes into play—namely, the high concentration of sodium lactate formed upon neutralization of

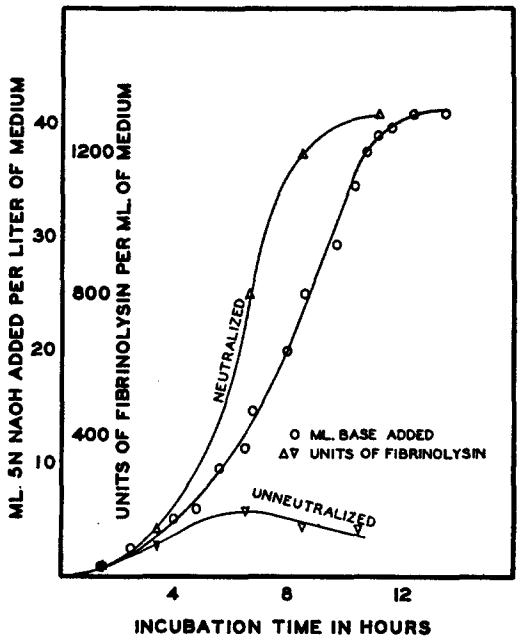

FIG. 1. Relation between acid production and fibrinolysin production in cultures of strain H46A, *Streptococcus hemolyticus*, group C. Acid production is expressed in terms of milliliters of 5N NaOH added to maintain neutrality of the culture. Fibrinolysin units are expressed as the reciprocal of the highest dilution lysing a standard fibrin clot in 30 minutes (Christensen, 1941).

the acid (Bernheimer and Pappenheimer, 1942). If the culture is not neutralized, not only are growth, acid production, and fibrinolysin production greatly diminished, but as time goes on, there is some destruction of preformed fibrinolysin. When the culture is kept neutral, however, fibrinolysin and acid production parallel each other, as shown in Fig. 1.

3. Fibrinolysin Solutions.—A 1:10 dilution of either the crude or partially purified concentrate was made up in gelatin buffer at pH 7.4. Experience has shown that this concentration is an appreciable excess in the usual test system.

4. Lysin Factor Solutions.—Milstone's (1941) procedure of diluting human serum with 19 volumes of distilled water containing 0.32 volume of 1 per cent acetic acid was

usually employed to precipitate the factor. Preparations of equal activity can be obtained by dialyzing the serum against distilled water and acidifying with acetic acid to pH 5.3. The most effective procedure when handling large volumes of serum was found to be precipitation of the factor by ⅓ saturation of the serum with ammonium sulfate. The precipitate was washed thoroughly with ⅓ saturated ammonium sulfate to remove other serum fractions carried down in the first precipitation. Whatever method of precipitation was used, the precipitate was collected by centrifugation and dissolved in a volume of saline buffer at pH 7.4 equal to $\frac{1}{10}$th the original serum volume.

5. Fibrinogen.—This was prepared by salting out with ammonium sulfate or sodium chloride and preserved by lyophilization as described in previous reports (Christensen and Jones, 1939, Christensen, 1940). Solutions of the desired concentration were made up in saline buffer, pH 7.4.

6. Thrombin.—Earlier, thrombin prepared from human plasma by the method of Tillett and Garner (1933) was used. Later, a commercial preparation of rabbit thrombin, hemostatic globulin,[1] was routinely employed in a dilution of 1:25 in saline buffer.

7. Casein.—Casein was prepared from fresh skim milk by acid precipitation followed by dehydration with alcohol and ether, according to the method described by Hawk and Bergeim (1937). For use, the dry powder was suspended in saline buffer and dissolved with the aid of alkali to make a 5 per cent solution at pH 7.4.

8. Gelatin.—Eastman purified calfskin gelatin was dissolved in saline buffer with the aid of heat to make a 4 per cent or 5 per cent solution at pH 7.4.

9. Saline Buffer.—M/10 KH_2PO_4 was adjusted to pH 7.4 with 5N base before making up to volume. The solution contained 0.9 per cent sodium chloride.

10. Gelatin Buffer.—Saline buffer containing 0.5 per cent Eastman purified calfskin gelatin, pH 7.4.

11. Acetate Buffer.—M/5 sodium acetate adjusted to pH 3.5 with M/5 acetic acid.

pH determinations on all solutions were carried out electrometrically, using a glass electrode.

All solutions containing organic reagents were preserved by the addition of 1:10,000 merthiolate. Madison and Snow's (1937) observation that merthiolate is without effect on the fibrinolysin reaction has been confirmed in this laboratory.

Determination of Lysin Factor-Fibrinolysin Activity

1. Fibrinolysis Time.—The technique used was essentially that of Tillett and Garner (1933), with minor modification of some details. The test contained 0.2 ml. of plasma or a solution containing an equivalent amount of fibrinogen, 0.1 ml. of fibrinolysin solution, and 0.2 ml. of rabbit thrombin (hemostatic globulin). Before addition of the thrombin solution, the volume was made up to 0.8 ml. with saline buffer to which was added any other reagents to be included in the test. After addition of the thrombin solution, the clots form within 1 minute. The tubes were incubated in a water bath at 37°C. Lysis time was taken as the time between formation and complete disappearance of the fibrin clot.

2. Decrease in Acid-Precipitable Nitrogen.—Aliquots of the lysin factor-fibrinolysin-

[1] The hemostatic globulin was kindly supplied by the Lederle Laboratories, Inc.

substrate mixture, consisting of 0.5 ml. of fibrinolysin solution, 0.5 ml. of lysin factor solution, and 6.0 ml. of substrate were blown into an equal volume of 10 per cent trichloracetic acid in Pyrex test tubes with conical tips. The mixture was allowed to stand for 15 to 30 minutes to ensure complete precipitation and the precipitates were packed by centrifugation. After washing with 5 per cent trichloracetic acid, the precipitates were digested in the same tubes and the nitrogen content determined.

3. Increase in Acid-Soluble "Tyrosine".—Anson and Mirsky (1932, 1933, 1937) have used a modification of Folin and Ciocalteu's (1927) phenol method to measure proteolysis. In this method, the acid-soluble tyrosine and tryptophane-containing split products of protein digestion are measured and expressed as liberated tyrosine. Recently, Heidelberger and MacPherson (1943) have improved the method by substituting sodium carbonate for sodium hydroxide and employing a Coleman spectrophotometer to measure the color developed. Based on these modifications, the analysis was carried out as follows: Aliquots of the reaction mixture were precipitated with an equal volume of 10 per cent trichloracetic acid. After flocculation, the precipitate was removed by filtration. 2.0 ml. of the filtrate were placed in a Pyrex tube, 6.0 ml. of 12.5 per cent sodium carbonate added, and the contents of the tube mixed. 1 ml. of the Folin and Ciocalteu phenol reagent, diluted 1:3, was added with shaking and the color allowed to develop for about 30 minutes. The color was read in a Coleman spectrophotometer at 650 mμ with the reagent blank set at 100 per cent transmittance. "Tyrosine" values were calculated from a calibration curve prepared with pure tyrosine.

4. Liberation of Amino Nitrogen.—Aliquots of the reaction mixture, usually 5 to 10 ml., were placed in a small beaker and adjusted to pH 7.0 with acid or alkali. Neutral formaldehyde (Merck reagent) was added so that the final concentration after titration would be between 6 and 9 per cent and the solution was titrated to pH 9.1 with 0.02N alkali, as recommended by Levy (1934). Titration was carried out electrometrically, using a Beckman electrometer with shielded glass electrode.

5. Decrease in Viscosity.—Equal volumes of fibrinolysin solution and lysin factor were mixed and incubated for 10 minutes to attain temperature equilibrium and allow activation of the lysin factor. 6 ml. of gelatin or other protein solution, warmed in the water bath to 37°C. were mixed with 1 ml. of the lysin factor-fibrinolysin solution and immediately poured into the pipette. Ostwald viscosity pipettes, with a flow time of between 15 and 24 seconds with distilled water at 37°C., were used. The water bath was maintained at 37 ± 0.1°C. The time of mixing the lysin factor-fibrinolysin mixture and the substrate solution was noted and the flow time of the reaction mixture determined at intervals of a few minutes, usually for a total period not exceeding 60 minutes.

6. Quantitative Determination of Proteolytic Activity.—The procedure used by Northrop (1932–33) to quantitate pepsin activity in terms of the per cent change in the specific viscosity of gelatin was adopted with slight modification. Northrop's equation for the calculation of pepsin units is as follows: $\frac{\Delta V\ 100}{V_0 \Delta T}$ = pepsin units, where V is the specific viscosity and is equal to $\frac{ts}{t_{H_2O}} - 1$, when ts is the flow time of the reaction mixture and t_{H_2O} is the flow time of distilled water in the pipette.

With the lysin factor-fibrinolysin system the reaction was carried out at pH 7.4 with 6 ml. of 4 per cent gelatin as the substrate and 1 ml. of the lysin factor-fibrinolysin mixture. The specific viscosity values obtained were plotted and a line drawn through the points. Activity was expressed in terms of proteolytic units (p.u.) and was calculated as $\frac{(V_0 - V_{10})\ 100}{V_0 \times 10}$ where V_0 is the initial specific viscosity and V_{10} is the viscosity at 10 minutes.

EXPERIMENTAL

Concentration and Partial Purification of Fibrinolysin

Fifteen or 30 liter lots of cultures of strain H46A, group C *Streptococcus hemolyticus* were cultivated by the methods described above. After growth had ceased, due to exhaustion of the glucose, the streptococcal cells were removed in a Sharples centrifuge. The supernatant, adjusted to pH 4.5 with glacial acetic or concentrated hydrochloric acid, was stored in the refrigerator. The following day the supernatant was concentrated to about $\frac{1}{10}$th the original volume in a large vacuum still at about 25°C. The syrupy concentrate was saturated with solid ammonium sulfate and allowed to stand at room temperature until flocculation was complete. A dark brown, flocculent precipitate formed and was collected on No. 50 Whatman paper by suction filtration. The precipitate, which contained practically all of the fibrinolytic activity of the original culture, was dissolved in saline buffer at pH 7.4 and centrifuged at high speed in a Swedish angle centrifuge to remove as completely as possible the cells and debris remaining after the original Sharples centrifugation. Since some fibrinolysin appeared to be adsorbed on the precipitate, the sedimented material was extracted 2 to 3 times with saline buffer and the extracts combined with the supernatant. The usual practice has been to maintain the active material as a precipitate in saturated ammonium sulfate solution in a volume representing a 150- to 200-fold concentration of the culture. In this form fibrinolysin is stable. Several preparations have shown no loss of activity in over 2 years when stored in the refrigerator.

Further purification can be accomplished by taking advantage of the fact that fibrinolysin is insoluble at a pH of 4.5 or below, while much inactive material is soluble. The crude fibrinolysin precipitate, prepared as described above, was dissolved in saline buffer with the aid of alkali in a concentration about 100 times that of the original culture. When acidified below pH 4.5 with concentrated acid, an abundant flocculent precipitate formed. This precipitate was washed with 200 ml. portions of acetate buffer, pH 3.5, until the washings were colorless. This procedure removed a considerable amount of color, derived mainly from the neopeptone in the medium, most of the residual phenol red, together with a considerable amount of inert, acid-soluble, nitrogenous material. The acid-extracted precipitate was dissolved in 100 ml. saline buffer at pH 7.4. The solution was dark brown in color, and still contained some insoluble material, chiefly bacterial cells, which could not be removed at this point by centrifugation or filtration because of the high viscosity of the solution. However, when this viscous solution was added to 5 to 10 volumes of alcohol, and the resulting precipitate redissolved in 100 ml. of saline buffer, the viscosity was reduced to such a degree that the insoluble debris could be spun out, leaving a dark brown, clear solution.

The insoluble precipitate was extracted twice with 100 ml. portions of saline buffer to elute adsorbed fibrinolysin, the extracts and supernatant pooled and precipitated by acidification. The precipitate was dissolved in saline buffer at pH 7.4 in a concentration 100 times that of the original culture. The nitrogen content of the solution was equivalent to a protein concentration of about 1 per cent, and represented about 4 per cent of the total nitrogen of the original culture medium. A flow chart of a typical preparation, with the nitrogen values of the material at the various stages is shown in Table I.

As indicated above, the fibrinolysin concentrate could not be freed from color by acid extraction nor could the color remaining after acid extraction be separated from the fibrinolysin by treatment with Norit charcoal, alumina gel, or by dialysis.

The partially purified material can be salted out by ammonium sulfate at concentrations between 22 and 40 per cent of saturation. Attempts to fractionate between these concentrations have resulted in smearing of the activity over the various fractions.

The fibrinolytic activity of the concentrated material, prepared in the manner just described, is only slowly destroyed by heating at 100°C at pH 7.4. It is stable at refrigerator temperature over a pH range of 3.0–8.5. It is readily destroyed, however, by treatment with trypsin and pepsin, indicating that the activity is associated with a protein.

Proteolytic Nature of Fibrinolysis

Twenty-five ml. of a solution of human fibrinogen precipitated by sodium chloride and equivalent to the fibrinogen in an equal volume of plasma were placed in a flask to which was added 20 ml. of a fibrinolysin concentrate which had been dialyzed ammonium sulfate-free, together with 5 ml. of a human thrombin solution. A second flask was prepared in a similar manner, except that saline buffer was substituted for thrombin. The flasks were incubated in a water bath at 37°C. and at intervals, 5 ml. portions of the contents of both flasks were removed and the trichloracetic acid-precipitable nitrogen determined. Zero time values were obtained by adding the reagents separately to the acid in order to avoid proteolysis.

From the data obtained in this experiment, as shown in Fig. 2, it is evident that the action of fibrinolysin on human fibrin and fibrinogen results in an appreciable decrease in acid-precipitable nitrogen. Furthermore, under the conditions of the experiment, there appears to be no significant difference between the hydrolysis of fibrin and fibrinogen.

It was felt desirable to confirm the evidence of proteolysis by other means. Since no differences could be detected between the hydrolysis of fibrin and fibrinogen, fibrinogen was used thereafter as a substrate in most experiments because the addition of another protein, thrombin, was unnecessary; and the substrate remained in solution throughout the experiment. Using a reac-

TABLE I

Concentration and Partial Purification of Streptococcal Fibrinolysin

30 liters neopeptone medium
(43.0 gm. nitrogen)

↓

Culture supernatant after removal of cells by centrifugation
(27.12 gm. nitrogen)

Adjust to pH 4.5 and concentrate to 4 liters at 25°C. *in vacuo*

↓

Saturate with ammonium sulfate and filter

Precipitate Dissolve in 300 ml. saline buffer, centrifuge	*Supernatant* (discard)
Supernatant Acidify supernatant and extracts with glacial acetic acid to pH 3.5–4.0, centrifuge	*Precipitate* Extract twice with saline buffer, discard precipitate, and combine extracts with supernatant
Precipitate Wash with acetate buffer until washings colorless, dissolve in 100 ml. saline buffer, pH 7.4 (1.04 gm. nitrogen) Pour into 1 liter alcohol at 0°C., centrifuge	*Supernatant* (discard)
Precipitate Dissolve in 300 ml. saline buffer, centrifuge	*Supernatant* (discard)
Supernatant Acidify with glacial acetic acid to pH 3.5–4.0, centrifuge	*Precipitate* Extract twice with saline buffer, combine extracts with supernatant
Precipitate Dissolve and make up to 300 ml. in saline buffer (0.87 gm. nitrogen)	*Supernatant* (discard)

tion mixture consisting of 12 volumes of a 5 per cent fibrinogen solution with 1 volume of lysin factor activated by 1 volume of fibrinolysin solution, the proteolytic nature of the reaction was confirmed by demonstrating the liberation of amino nitrogen, increase in acid-soluble "tryrosine," and decrease in the viscosity of the mixture, as shown in Fig. 3. If lysin factor is not added to the reaction mixture, proteolysis occurs but to a lesser extent. This is due in all likelihood to the presence of lysin factor in the fibrinogen solution, since as stated above, no attempt was made to prepare the fibrinogen free from lysin factor.

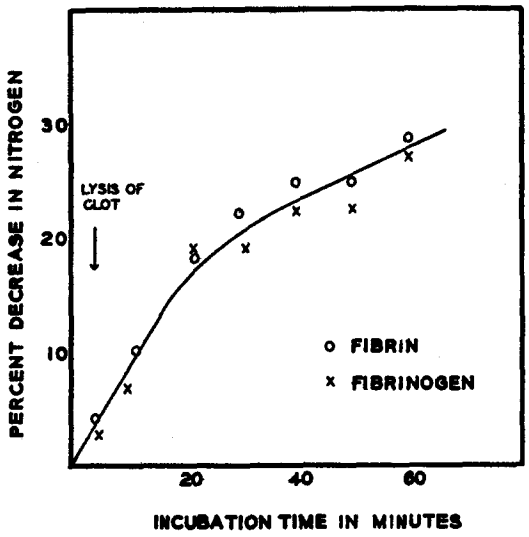

FIG. 2. Decrease in acid-precipitable fibrin and fibrinogen nitrogen following treatment with fibrinolysin. Results are expressed as per cent decrease in the nitrogen found in the precipitate.

Rôle of Lysin Factor

With definite evidence that fibrinolysis and fibrinogenolysis involve proteolysis of the substrate, it became of interest to ascertain the rôle of lysin factor in the reaction. Milstone (1941) has shown that both fibrinogen and thrombin, as ordinarily prepared, are contaminated with lysin factor. Clots prepared from fibrinogen and thrombin purified by Milstone's method usually do not lyse when treated with unconcentrated culture filtrates as a source of fibrinolysin. However, when these clots are treated with concentrated fibrinolysin, lysis usually occurs. Because of the labor involved in purifying fibrinogen, and because it proved extremely difficult to prepare a product which would not lyse in the presence of highly concentrated preparations of fibrinolysin, other substrates were sought which would be free of lysin factor.

1. Lysis of Rabbit Fibrin.—Milstone (1941) found that rabbit plasma clots do not lyse on the addition of active culture filtrates unless human lysin factor is also added. These results were interpreted by Milstone as indicating the lack of a suitable lysin factor in rabbit serum. When attempts were made to repeat these experiments with concentrated fibrinolysin, it became evident that the difference between human and rabbit plasma clots is not due to lack of lysin factor in the latter. With concentrated fibrinolysin, rabbit plasma clots

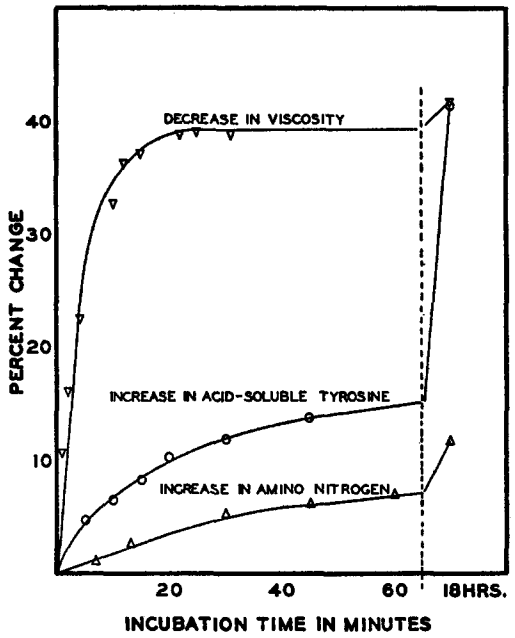

FIG. 3. Proteolysis of fibrinogen by lysin factor-fibrinolysin. The decrease in viscosity is expressed as the per cent decrease from the initial relative viscosity; acid-soluble "tyrosine" and amino nitrogen are expressed as the per cent of total substrate "tyrosine" and nitrogen respectively.

undergo lysis readily, whereas on dilution of the fibrinolysin to approximately the concentration of the average culture filtrate, the clots do not lyse unless human lysin factor is added. The following experiment illustrates this finding. Duplicate sets of standard fibrinolysin tests were prepared with various dilutions of a fibrinolysin concentrate. Two-tenths ml. of human lysin factor solution was added to one series, an equal volume of saline buffer to the other. The tubes were placed in the water bath and the lysis time of the clots in the presence and absence of lysin factor noted. The data obtained are shown in Table II.

It is evident from the results shown in Table II, that while dilute solutions of

fibrinolysin do not cause lysis of rabbit plasma clots in the absence of added lysin factor, fibrinolysis takes place when concentrated fibrinolysin solutions are used.

The resistance of rabbit fibrin may be due to the presence of an inhibitor, in addition to a deficiency of lysin factor, as suggested by Milstone (1941). Evidence supporting this view was obtained in experiments which showed that addition of rabbit serum to human fibrin clots causes a prolongation of lysis time. As shown in Table III, when varying amounts of rabbit serum are added to the test system containing human fibrinogen clotted with thrombin in the presence of a constant amount of fibrinolysin, the higher concentrations of

TABLE II

Lysis of Rabbit Fibrin with Concentrated Fibrinolysin

Dilution of fibrinolysin	Lysis time	
	With lysin factor	Without lysin factor
	min.	*min.*
Undiluted	1.5	10.0
1:10	1.5	16.5
1:40	1.5	Overnight*
1:160	3.5	No lysis‡
1:640	14.0	" "
1:2560	Overnight	" "
Control	Negative	" "

* Indicates no lysis observed during the first day's observation. Clots found lysed the following morning.

‡ No lysis observed in 24 hours.

rabbit serum produce a significant prolongation of the time required for fibrinolysis.

The presence of an inhibitor in rabbit plasma is also indicated by the following observations. Fibrinogen was isolated from a sample of rabbit plasma by salting out with sodium sulfate (fibrinogen I). A portion of this fibrinogen was further purified by a second precipitation with sodium sulfate (fibrinogen II). The two preparations were dissolved in saline buffer in a volume equal to the volume of the plasma from which they were isolated. Fibrinolysin tests were set up with these preparations, each containing, in addition to the fibrinogen or plasma, 0.2 ml. human lysin factor, 0.1 ml. of the fibrinolysin dilution, and 0.2 ml. of hemostatic globulin, diluted 1:25. The results of these tests are shown in Table IV.

As may be seen from the data presented in Table IV, purification of rabbit fibrinogen by reprecipitation results in a decrease in the lysis time of fibrin clots prepared from it, suggesting the removal of an inhibitor. This is most apparent in the tests in which the two highest dilutions of fibrinolysin were used.

2. Proteolysis of Casein.—This protein, similar to fibrinogen in that it is clotted by a specific enzyme, was tested as a possible substrate. One ml. of skim milk was clotted by rennin in the presence of fibrinolysin alone, lysin factor alone, and fibrinolysin and lysin factor together. The solutions were placed in a water bath at 37°C. and examined at intervals for evidence of lysis. The results are shown in Table V.

TABLE III

Inhibition of Lysis of Human Fibrinogen by Rabbit Serum

Rabbit serum dilution	Lysis time of clots
	min.
Undiluted	120.0
1:10	6.0
1:100	4.5
1:1000	4.5
Control	4.5

Dilutions of rabbit serum in a volume of 0.1 ml. were added to the fibrinolysin test containing human fibrinogen clotted by hemostatic globulin in the presence of 0.1 ml. of fibrinolysin concentrate diluted 1:10.

TABLE IV

Effect of Fibrinogen Purification on Lysis Time of Rabbit Fibrin

Fibrinolysin dilution	Lysis time of clots prepared from		
	Whole plasma	Fibrinogen I	Fibrinogen II
Undiluted	—	No clot*	No clot
1:10	1.5 min.	" "	1.5 min.
1:40	1.5 "	1.5 min.	1.5 "
1:160	3.5 "	5.5 "	3.5 "
1:640	14.0 "	16.0 "	8.0 "
1:2560	Overnight‡	67.0 "	15.5 "

* A fibrin clot never formed. This was due to destruction of the fibrinogen before clotting could occur, and is not infrequently seen with highly active fibrinolysin preparations.

‡ No lysis observed during the first day, clots found lysed the next morning.

As shown in Table V, neither fibrinolysin nor lysin factor alone had any effect on the casein clot. However, when both were present, "caseinolysis" analogous to fibrinolysis, occurred.

With evidence that casein is susceptible to the action of the lysin factor-fibrinolysin system, experiments similar to those with fibrinogen were carried out to determine the extent of proteolysis.

One volume of fibrinolysin solution and 1 volume of lysin factor were mixed and incubated for 10 minutes at 37°C. Twelve volumes of 5 per cent casein

solution were then added, mixed thoroughly, and incubation at 37° continued. Determination of acid-soluble "tyrosine," amino nitrogen, and viscosity of the

TABLE V

"Caseinolysis" by Lysin Factor-Fibrinolysin

Skim milk	Lysin factor	Fibrinolysin	Rennin	Lysis time
ml.	*ml.*	*ml.*	*ml.*	*min.*
1.0	0.5	0.5	0.1	30–60
1.0	0.5	—	0.1	No lysis
1.0	—	0.5	0.1	" "

Milk was freed of fat by centrifugation. The rennin solution was prepared by dissolving one Chr. Hansen junket tablet in 10 ml. distilled water. The fibrinolysin concentrate was diluted 1:10 in saline buffer.

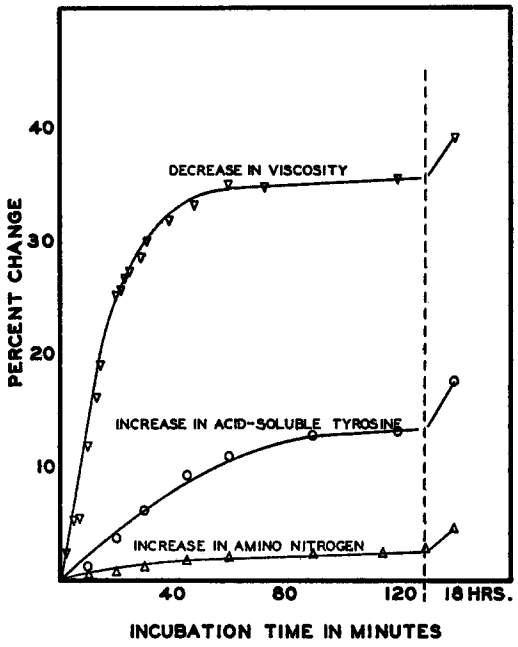

FIG. 4. Proteolysis of casein by lysin factor-fibrinolysin. Experimental details identical with those of Fig. 3, except that the substrate was 5 per cent casein instead of fibrinogen.

mixture at various time intervals showed that casein is susceptible to the proteolytic action of the lysin factor-fibrinolysin system. The results of this experiment are shown in Fig. 4.

3. Proteolysis of Gelatin.—A 5 per cent gelatin solution was tested for susceptibility to the action of the lysin factor-fibrinolysin system under the same

experimental conditions as in the case of fibrinogen and casein. Liberation of amino nitrogen and decrease in the viscosity of the mixture, as shown in Fig. 5, indicate that gelatin is also hydrolyzed.

The experiments with casein and gelatin support Milstone's (1941) hypothesis that both fibrinolysin and lysin factor must be present to constitute an active system. No hydrolysis of either casein or gelatin could be detected in the present experiments when either fibrinolysin or lysin factor was used alone, but only when both reagents were present.

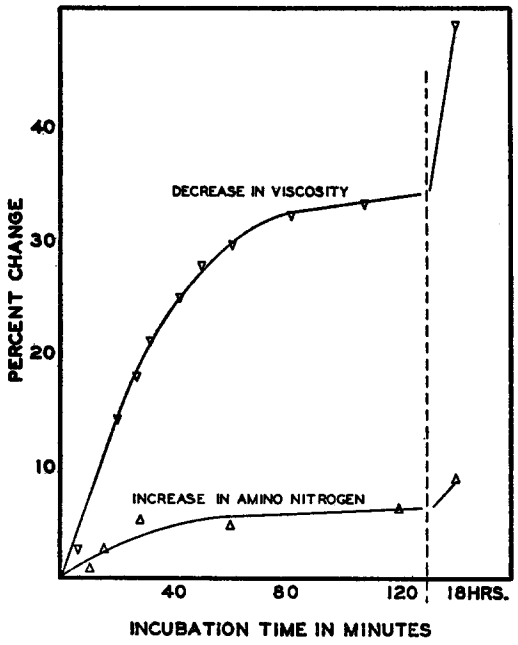

FIG. 5. Proteolysis of gelatin by lysin factor-fibrinolysin. Experimental details identical with those of Figs. 3 and 4, except for the substitution of gelatin as substrate.

Nature of the Interaction between Lysin Factor and Fibrinolysin

Since both fibrinolysin and lysin factor are necessary for activity, two possibilities may be suggested to explain their interaction. On the one hand, lysin factor may activate fibrinolysin, the latter being the proteolytic enzyme, or alternatively, fibrinolysin activates lysin factor and renders it proteolytic. The experiments to be detailed below indicate that in all probability lysin factor is the proteolytic agent which is activated by fibrinolysin.

1. Spontaneous Activation of Lysin Factor.—Samples of lysin factor which have been stored in the cold, after a variable period may develop proteolytic activity spontaneously. The proteolytic activity of a number of batches of

lysin factor was determined shortly after preparation and at intervals thereafter, both in the presence and absence of fibrinolysin. The activity of four of these samples, prepared by both dilution and acidification and by salting out, is shown in Table VI.

From the data presented in Table VI, it can be seen that in all instances, the addition of fibrinolysin to lysin factor which had become active spontaneously did not result in the development of more proteolytic activity than when fibrinolysin was added to the lysin factor before the latter had developed spontaneous activity.

If spontaneous activity of the aged lysin factor preparations were due to the presence of another proteolytic enzyme, it might be expected that the total

TABLE VI

Spontaneous Activation of Lysin Factor

Lysin factor No.	Date tested	Activity in proteolytic units	
		With fibrinolysin	Without fibrinolysin
253	5/26/43	0.7	0
	6/2/43	0.7	0
	8/2/43	0.7	0.6
406	9/16/43	1.3	0
	11/3/43	0.7	0.6
513	2/12/44	2.4	0
	6/9/44	2.6	2.3
539	5/27/44	1.7	0
	6/9/44	1.7	1.8

Activity expressed in terms of proteolytic units in 0.5 ml. of lysin factor solution, determined by the procedure described in the section on Methods.

activity of the aged preparation, when treated with fibrinolysin would be equal to the sum of the original lysin factor-fibrinolysin activity plus the activity developed on ageing, but in no instance has such an increase in the total activity of the system been noted.

On the other hand, of numerous samples of fibrinolysin, none has ever shown any proteolytic activity in the absence of lysin factor, when tested on the lysin factor-free substrates, casein and gelatin.

2. Catalytic Nature of Lysin Factor Activation.—When serial dilutions of lysin factor are mixed with an excess of fibrinolysin and incubated, maximum proteolytic activity develops in less than 10 minutes, and is proportional to the concentration of lysin factor. The results of such an experiment are shown in Table VII.

On the other hand, when dilutions of fibrinolysin are mixed with constant amounts of lysin factor and incubated, the activity developed is proportional

not to the concentration of fibrinolysin, but to the incubation time of the lysin factor-fibrinolysin mixture. In other words, the total proteolytic activity

TABLE VII

Relation between Lysin Factor Concentration and Activity

Lysin factor dilution	Activity in proteolytic units
Undiluted	3.1
1:2	1.8
1:4	0.7
1:6	0.6
1:8	0.4

Lysin factor solution in a volume of 0.5 ml. was activated by treatment with 0.5 ml. of 1:10 dilution of concentrated fibrinolysin for 10 minutes at 37° C. The activated lysin factor-fibrinolysin mixture was then mixed with 6 ml. gelatin, warmed to 37°, and the activity determined.

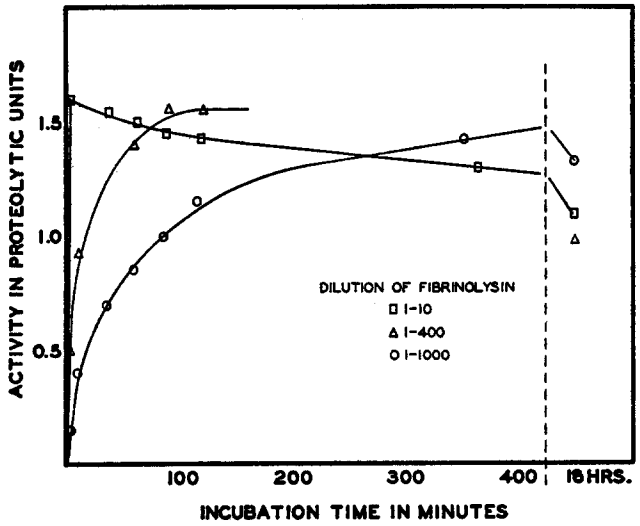

FIG. 6. Activation of lysin factor by fibrinolysin. Aliquots of lysin factor solution mixed with an equal volume of fibrinolysin dilutions and incubated at 25°C. At intervals, 1 ml. of each mixture removed and tested for activity by the procedure described under Methods.

developed with a constant amount of lysin factor and different amounts of fibrinolysin ultimately reaches the same level in each case, given a sufficiently long incubation period, as shown in Fig. 6. Furthermore, with a constant amount of lysin factor, the rate of activation is proportional to the fibrinolysin concentration.

These experiments indicate that lysin factor is activated by fibrinolysin and also that the activation is not due to a stoichiometric reaction between the two, but is catalytic in nature.

DISCUSSION

It is evident from the results of the experiments described in this paper that streptococcal fibrinolysis is a proteolytic reaction, the active proteolytic enzyme being produced as the result of an interaction of streptococcal fibrinolysin and the lysin factor normally present in plasma and serum. The dissolution of the fibrin gel, heretofore considered the characteristic feature of the fibrinolytic reaction, appears to be merely an early manifestation of proteolysis. It has also been shown that proteolysis is not limited to fibrin and fibrinogen, since in addition to these substrates, casein and gelatin are also susceptible to the action of the lysin factor-fibrinolysin system. Failure on the part of earlier workers to demonstrate an action of streptococcal fibrinolysin on proteins other than fibrin and fibrinogen was undoubtedly due to the fact that the part played by the serum component, lysin factor, was not recognized until the work of Milstone.

The essential rôle of lysin factor in the proteolytic reaction is most clearly demonstrated in the experiments with proteins other than fibrin and fibrinogen. The results of experiments with fibrinogen and thrombin must always be interpreted with caution because of the great difficulty in removing all traces of lysin factor from these reagents. As indicated in the experimental section, fibrinogen can be prepared sufficiently free from lysin factor that dilute solutions of fibrinolysin will not lyse fibrin clots prepared from it. Lysis of such fibrin clots on the addition of highly concentrated fibrinolysin might be interpreted as being due to a proteolytic action of the fibrinolysin itself. When highly concentrated fibrinolysin is mixed with other proteins, however, for example casein and gelatin, no trace of proteolysis has ever been detected unless lysin factor was also added. It appears, therefore, that the interaction of fibrinolysin and lysin factor is necessary for the production of the proteolytic agent, and that the slight susceptibility of purified fibrin clots to fibrinolysin is due to the presence of small amounts of lysin factor not removed during purification.

It has been assumed that the relative or absolute resistance of animal fibrins to streptococcal fibrinolysin is due to species differences in the fibrin itself. Tillett and Garner (1933) and Madison (1934–35), however, showed that "hybrid" fibrins are susceptible if one of the components, either fibrinogen or thrombin, is of human origin. These results were interpreted by Milstone (1941) as indicating a deficiency of a suitable lysin factor in the plasma of the resistant species, a lack which might be supplied by the human component of the system. Actually, as shown by the present data, rabbit fibrin clots are susceptible to lysis if sufficient fibrinolysin is used in the system. Additional evidence

that rabbit plasma contains lysin factor is indicated by the observation that successive precipitations of rabbit fibrinogen render the fibrin clots progressively more susceptible to the action of fibrinolysin.

The anomalous behavior of rabbit plasma as compared with human may be due to several factors. The present experiments indicate that rabbit serum contains an inhibitor of the proteolytic enzyme, which may account in part for the resistance of rabbit plasma clots. However, the presence of an inhibitor in these clots does not explain fully the susceptibility of "hybrid" clots. It is also possible therefore, that rabbit serum contains less lysin factor than does human, or that lysin factor in rabbit serum is qualitatively different and is activated by fibrinolysin at a slower rate than human lysin factor.

A plausible explanation for the necessity of interaction between lysin factor and fibrinolysin before the appearance of proteolytic activity is that one of these components activates the other. In the presence of excess fibrinolysin, the proteolytic activity developed is directly proportional to the concentration of lysin factor in the system, hence it appears that lysin factor is the component which is converted to a proteolytic enzyme. The validity of this hypothesis is also borne out by the fact that lysin factor preparations may develop proteolytic activity spontaneously, whereas in our experience fibrinolysin solutions never do. In addition, treatment of a constant amount of lysin factor with varying amounts of fibrinolysin results in the eventual liberation of an equal amount of proteolytic activity in each instance, and the rate of activation is proportional to the fibrinolysin concentration. It may be suggested, therefore, that lysin factor represents an inactive proteolytic enzyme or "zymogen" of serum which is activated by fibrinolysin. The analogy between this system and the trypsinogen-enterokinase and chymotrypsinogen-trypsin systems is striking. The lysin factor system is more nearly analogous to the chymotrypsinogen system, however, in that activation does not appear to be rapidly autocatalytic as is the case with the trypsinogen-trypsin system. It is not suggested, however, that lysin factor is chymotrypsinogen, since the two have different pH optima and differ in other respects.

Activation of a proteolytic enzyme from an animal source by a kinase derived from a microorganism is not without precedent, since Kunitz (1938) has shown that trypsinogen may be activated by a kinase obtained from cultures of a mold of the genus *Penicillium*.

It has been known for many years that human and animal sera contain a proteolytic enzyme normally present in an inactive state. In a recent series of papers, Tagnon and coworkers (1942) have reviewed much of the literature concerning this enzyme. Certain similarities between lysin factor and the serum protease are apparent. For example, both are found in the globulin fraction of serum; both are active at neutrality; both digest casein and gelatin as well as fibrin; and both are present in serum in an inactive state. As originally shown

by Nolf (1908), the serum protease is activated by treatment of the serum with chloroform. Lysin factor, on the other hand, is activated by streptococcal fibrinolysin. It is possible, however, that this difference merely represents two methods of activating the same enzyme.

Based on the evidence presented in this paper, the following hypothesis of streptococcal fibrinolysis is advanced. Lysin factor is an inactive proteolytic enzyme or "zymogen" in serum which is activated by a kinase, streptococcal fibrinolysin. Addition of fibrinolysin to a solution containing lysin factor results in the catalytic conversion of the lysin factor to an active proteolytic enzyme, which is able to cause fibrinolysis of fibrin clots or proteolysis of other proteins such as casein or gelatin. Demonstration of the action of streptococcal fibrinolysin and lysin factor on substrates other than fibrin or fibrinogen makes it obvious that the specific designation "fibrinolysin" for this component of streptococcal culture filtrates is inaccurate.

SUMMARY AND CONCLUSIONS

1. Methods for the preparation and partial purification of streptococcal fibrinolysin are described.

2. The lysis of fibrin clots in the presence of streptococcal fibrinolysin is associated with proteolysis of the fibrin. Digestion is due to an enzyme normally present in serum or plasma in an inactive state, which is activated by fibrinolysin. Fibrinolysin alone has no demonstrable proteolytic activity.

3. The lysin factor-fibrinolysin system brings about proteolysis of other proteins such as gelatin or casein, in addition to fibrin and fibrinogen.

4. It is suggested that lysin factor exists in serum or plasma as a zymogen, and that it is activated by fibrinolysin, a kinase, in a manner similar to the activation of trypsinogen by enterokinase or the mold kinase of Kunitz (1938).

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