BIOCHEMICAL STUDIES ON THE FIBRINOLYTIC ACTIVITY OF HEMOLYTIC STREPTOCOCCI

I. Isolation and Characterization of Fibrinolysin

By R. L. GARNER,* Ph.D., and WILLIAM S. TILLETT, M.D.

(*From the Biological Division of the Department of Medicine, The Johns Hopkins Medical School, Baltimore*)

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The fibrinolytic activity of hemolytic streptococci designates the capacity of cultures of the organisms to liquefy rapidly human fibrin clot (1). The lytic action is referable to the presence in the culture of an extracellular substance, characteristically elaborated by those strains of hemolytic streptococci which are derived from patients suffering with acute streptococcus infections.

Investigation of the phenomenon has included studies concerning the mechanism involved in the reaction and consideration of a possible relationship to the more general problem of infection and resistance (2).

The biochemical studies to be described in this and the succeeding communication deal with two aspects of the phenomenon. This report is concerned with the isolation and characterization of the active fibrinolytic principle elaborated by the organisms. Methods are described by which the lytic substance may be removed from culture filtrates, concentrated, and partially purified. Some of the physical properties of the active preparations are described.

The second paper (3) deals with the biochemical action of the lytic substance and the nature of the end-products.

The phenomenon of fibrinolysis induced by the bacterial agent consists of a rapid change of fibrin from its solid clotted form to a soluble substance. The transformation, therefore, as it occurs *in vitro*, is readily visible, since the coagulated gel is not disturbed by inversion of the test tubes and the process of liquefaction converts the

* John D. Archbold Fellow in Medicine, Department of Medicine.

clot to a limpid solution. The end-point of a fibrinolytic test is established by determining the occurrence of complete dissolution.

Throughout the study, the length of time necessary to effect liquefaction is employed as a rough quantitative measure of the amount of fibrinolytic substance present in a test preparation of bacterial material. For example, when one sample liquefies a given amount of fibrin in 2 minutes and another sample requires 1 hour to dissolve the same amount of fibrin, this difference in time is used as a relative measure of fibrinolysin in the two preparations. Furthermore, when 0.001 cc. of one sample causes lysis just as quickly as does 0.5 cc. of another sample, the former preparation is deemed to contain more fibrinolytic substance than the latter. The complications involved in measuring quantity on the basis of time are appreciated but the method is, nevertheless, applicable to the purpose of the experiments which follow.

Materials and Methods

In the earlier report (1) it was demonstrated that when fibrinogen, chemically isolated from human plasma, was clotted with preparations of thrombin, also derived from human blood, lysis occurred with extraordinary rapidity. For the purposes of the experiments with which this study is concerned, it has been advantageous to use fibrinogen and thrombin solutions, thus eliminating some of the fractions of whole blood, which do not participate in the reaction.

It has also been demonstrated (1) that, even though rabbit plasma clot is resistant to dissolution, rabbit fibrinogen, when coagulated with human thrombin, forms a fibrin substrate which is highly susceptible to lysis. Rabbit fibrinogen-human thrombin combinations have been found to be uniformly satisfactory in tests concerning the activity of the fibrinolytic substance and have been regularly employed for this purpose.

Fibrinogen and Thrombin Solution.—The fibrinogen and thrombin solutions were prepared according to the methods described in the earlier report (1).

Cultures.—A strain of *beta* hemolytic streptococcus, designated Co, has been employed in these studies. The organism was grown in beef infusion broth which contained 2 per cent NaCl, 1 per cent peptone, and 0.05 per cent dextrose. The broth was adjusted to pH 7.6 but did not contain additional buffers. Incubation of the culture was carried out for 14 to 18 hours. At the end of the incubation period the culture was centrifuged and the supernatant fluid was filtered through a Berkefeld Type V candle. The sterile filtrate was then tested for fibrinolytic activity in the manner described below. Only highly potent material was used.

Description of Test.—Titration consisted in testing the potency of progressive dilutions of material containing streptococcal fibrinolysin. The test for the fibrinolytic potency of a preparation, unless otherwise stated, was carried out as

follows: To a series of appropriate dilutions of the test solution sufficient physiological saline solution was added to bring the volume of each to 0.7 cc. 0.2 cc. of a solution of rabbit fibrinogen was added and thoroughly mixed. Then 0.1 cc. of a human thrombin preparation was added as rapidly as possible after the introduction of fibrinogen, and each tube was well shaken. The test was then placed in the water bath at 37.5°C. and the time of coagulation and liquefaction of each clot was recorded. When fresh preparations were employed the coagulation time, determined in the usual manner by inverting the tube, rarely exceeded one minute. All solutions were warmed to room temperature prior to using them in a test.

Other technical procedures are given in the body of the report according to the type of experiment which was performed.

Isolation, Concentration, and Partial Purification of the Fibrinolysin of Hemolytic Streptococci

The fact that the streptococcal fibrinolysin is copiously present in the cell-free Berkefeld filtrates of broth cultures of hemolytic streptococci has greatly facilitated studies of the active principle. In the experiments to be described, therefore, highly active, sterile filtrates were always utilized, thus excluding the possible complications associated with the growth and metabolism of living cells.

Four methods have been used for the purpose of isolating and concentrating the active fibrinolytic principle. They are: (*a*) concentration under reduced pressure; (*b*) precipitation with organic solvents; (*c*) concentration by vacuum dialysis; (*d*) specific adsorption and elution.

(*a*) *Concentration under Reduced Pressure.*—400 cc. of active culture filtrate were concentrated *in vacuo*. During the distillation the water bath was maintained at 40°C., and the vacuum was obtained with an oil pump. The receiver was cooled in an ice-salt bath. Under these conditions the volume of filtrate was reduced to 40 cc. in 4 hours at which time the operation was terminated. The tenfold concentrate, which was deeply colored, was then tested for fibrinolytic activity and its potency compared with that of the original solution. The results are as follows:

Dilution of solution, *cc.*	0.5	0.1	0.05	0.01	0.005	0.0001	0.0005
	min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
Original filtrate	2*	5	10	31	45	—	—
Concentrate	2	2	2	9	27	40	63
Concentrate (after 24 hrs.)	5	7	45	—	—	—	—

* In the tabulations, figures indicate not only that dissolution occurred but also the number of minutes required for lysis; the symbol (—) indicates that no lysis occurred in 2 hours.

From these results it can be seen that not only has the concentrate retained its activity after the treatment but that a definite increase, at least fivefold, has been obtained. The potency of the preparation did not remain constant and an appreciable decrease in titer was observed after storage for even so short a time as 24 hours in the ice box. Although the results of these preliminary experiments indicate that concentration may be effected under reduced pressure, the instability of the product prepared in this manner has rendered its use impractical. Consequently, the method has not been regularly employed.

(*b*) *Precipitation with Organic Solvents.*—The fibrinolytic principle, together with other organic materials and inorganic salts, was precipitated when the culture filtrate was treated with several volumes of either acetone, methyl alcohol, or ethyl alcohol. The latter precipitant has been regularly employed. The procedure was as follows:

Active culture filtrate, 750 cc., was cooled to 0°C. in an ice-salt bath. Three volumes of cold 95 per cent alcohol (2,250 cc.) were slowly added with constant stirring. The mixture was permitted to stand at 0°C. for an hour. The material readily flocculated and was collected by centrifugation. The grey colored precipitate was stirred with ice-cold absolute alcohol, transferred to a small Buchner funnel, and washed alternately with cold absolute alcohol and cold anhydrous ether. After a final washing with ether the amorphous material was dried in a vacuum desiccator which contained phosphoric anhydride and chips of paraffin. When thoroughly dry, the material was ground to a fine powder and was stored over $CaCl_2$. The yield was 1.88 gm. of solid substance from 750 cc. of culture filtrate, or approximately 2.5 mg. per cc.

The dry preparation of the fibrinolytic substance dissolved slowly but completely in physiological saline and in dilute acid pH 6.0. Solution in M/20 phosphate buffer, pH 8.0, left a small amount of insoluble residue which was, however, shown to contain none of the fibrinolytic principle. A solution prepared by dissolving 2.5 mg. of the dry powder in M/20 phosphate-buffered physiological saline, pH 7.2, was tested for fibrinolytic activity and compared to the original filtrate. In addition, a more concentrated solution containing 10 mg. per cc. was prepared and tested. The results were as recorded:

Dilution of solution, *cc.*	0.5	0.05	0.005	0.0025	0.001
	min.	*min.*	*min.*	*min.*	*min.*
Original filtrate	6	5	21	—	—
Dry preparation, 2.5 mg. per 1 cc	3	9	34	—	—
Dry preparation, 10 mg. per 1 cc	2	5	20	41	75

From these results it is seen that practically all of the fibrinolysin is precipitated by treatment of active culture filtrates with alcohol. The activity of the dry preparations is readily demonstrated by testing appropriate solutions of the precipitate. Thus, a solution which contained the dry material in a concentration approximately equivalent to that of the original culture filtrate (calculated upon the basis of the yield) compared favorably with the latter in respect to fibrinolytic potency. A more concentrated solution of the dry material was proportionally more potent in promoting the dissolution of the fibrin clots. Since the solubility of such a dry preparation is relatively low, the precipitation method has not proved to be of particular value from the point of view of the concentration of the fibrinolysin. The method is useful, however, as a means of obtaining a quantity of active material for preservation in the dry state. The stability of the preparations is shown by the fact that material stored for 11 months in a desiccator at room temperature lost none of its original potency.

Although consistently highly active, the more concentrated solutions of the dry preparations have occasionally inhibited the coagulation of plasma by $CaCl_2$. This inhibition may be attributable to the presence, in the fibrinolytic preparation, of peptone, which has been variously reported to possess anticoagulant properties. Many preparations have been obtained, however, not having an anticoagulant effect.

(*c*) *Concentration by Vacuum Dialysis.*—Active culture filtrate, 750 cc., was placed in a vacuum dialyzer similar to the apparatus described by Simms (4). Dialysis against distilled water under a negative pressure of 40 cm. of mercury was permitted to proceed for 20 hours. During this time the volume of the filtrate was reduced to 25 cc. The solution was tested for fibrinolytic activity, and compared with the original filtrate. The results were as follows:

Dilution of solution, *cc.*	0.5	0.05	0.005	0.0005	0.0001
	min.	*min.*	*min.*	*min.*	*min.*
Original filtrate	7	8	90	—	—
Concentrated	2	2	4	27	76
Concentrated (7 days old)	2	2	4	38	95

As can be readily seen, concentration was even more successful by this method than by the use of reduced pressure (vacuum distillation).

Furthermore, the solution, stored in the ice box for a week, did not decrease appreciably in titer. The stability of the solution over a longer period was not tested.

(*d*) *Specific Adsorption and Elution.*—A variety of inert materials were tested for the property of specific adsorption of the fibrinolytic principle from culture filtrates. Among these kaolin, talcum, charcoal, and the diatomaceous earths were without effect. It was possible, however, to obtain specific adsorption of the active principle upon alumina. The polyaluminum hydroxide B of Willstätter (5) has proved of particular value for this purpose.

Active culture filtrate, 2,500 cc., was intimately mixed with the aluminum hydroxide gel prepared from 250 gm. of aluminum sulfate ($Al_2(SO_4)_3 \cdot 18\ H_2O$). The mixture was incubated at 37.5°C. for an hour, during which time it was frequently stirred. The alumina was removed by centrifugation. That adsorption had taken place was indicated by the fact that the supernatant fluid, when properly tested, possessed no fibrinolytic activity. The alumina was then suspended in 1,000 cc. of physiological salt solution, thoroughly stirred, and centrifuged. The supernatant saline solution contained a considerable amount of pigment but was shown by test to have extracted none of the fibrinolytic principle. The washing of the gel was repeated twice with physiological salt solution and twice with distilled water. The alumina was then thoroughly triturated with 500 cc. of a M/10 phosphate buffer solution which was prepared by dissolving 35.8 gm. of $Na_2HPO_4 \cdot 12\ H_2O$ in 950 cc. of distilled water, adjusting the pH to 7.3 by the careful addition of a little concentrated HCl, and diluting to a volume of 1,000 cc. The alumina suspension was incubated for 1/2 hour at 37.5°C. and was centrifuged. The supernatant fluid was highly active in promoting the dissolution of the fibrin. The second and third elutions were carried out in the same manner. The fourth elution fluid contained only a trace of the fibrinolytic active substance and was discarded. The first, second, and third elution fluids were combined and filtered through a Berkefeld filter. The clear, highly active eluate contained in 1,500 cc. was then concentrated in the Simms dialyzer to a volume of 50 cc. Since the dialysis required approximately 16 hours, sterility was insured by the addition of a little toluene before beginning the procedure. The resulting clear, slightly brown concentrate was tested for fibrinolytic potency and compared to the original culture filtrate.

A 5 cc. portion of the concentrate was evaporated to dryness *in vacuo* over phosphoric anhydride. The product, dark brown scales, dissolved readily in 5 cc. of physiological salt solution and was tested for the fibrinolytic activity in the usual manner. A summary of the tests carried out upon the various solutions which were obtained during the experiment is presented below:

Preparation No.	Material, *cc.*	0.5	0.05	0.005	0.001	0.0005	0.0001
		min.	*min.*	*min.*	*min.*	*min.*	*min.*
I	Original filtrate	7	8	36	—	—	—
II	Preparation I, adsorbed	—	—	—	—	—	—
III	Saline washing	—	—	—	—	—	—
IV	Elution 1	2	4	15	50	—	—
V	Elution 2	2	2	8	34	63	—
VI	Elution 3	6	9	41	—	—	—
VII	Elution 4	15	—	—	—	—	—
VIII	Combined Elutions 1, 2, 3	2	2	8	37	54	—
IX	Preparation VIII; concentrated	2	2	5	16	23	64
X	Preparation IX; stored 14 days	2	2	7	20	35	50
XI	Preparation IX; dried, redissolved	2	2	6	19	25	55

The adsorption of the fibrinolysin upon the alumina and the application of appropriate elution and concentration processes resulted in the most potent preparations of the fibrinolytic principle which have thus far been obtained. Although the method of comparing the solutions by titer cannot be considered accurately quantitative, inspection of the results indicates that the various steps in the procedure may be performed with but little loss of the active material originally present in the filtrate.

Throughout the experimental work which has been described it was frequently observed that different culture filtrates varied in their content of fibrinolysin. The difference was not apparent when 0.5 cc. of the filtrate was tested but became increasingly manifest when higher dilutions of the active filtrate were used. The fact becomes important when the potencies of the various preparations of the fibrinolysin are to be compared. An experiment was therefore carried out in which the three most favorable methods of concentration were applied to portions of the same culture filtrate. The results of the experiment are presented in Table I.

The above experiment, which was carried out with a single culture filtrate, leaves no doubt that the active fibrinolytic principle of hemolytic streptococci may be successfully concentrated.

Properties of the Fibrinolytic Principle

Effect of Heat on Fibrinolysin.—The resistance of the streptococcal fibrinolysin to thermal inactivation has been found to be conditioned

by several factors relating to the test material. The experiments have, therefore, been carried out not only with culture filtrates, but also with concentrated preparations isolated by the methods just described. A summary of the results of a series of heating experiments is presented in Table II.

TABLE I

Comparison of Different Preparations of the Fibrinolytic Principle from Culture Filtrate 110

No.	Preparation	Amount of preparation									
		0.5 cc.	0.1 cc.	0.05 cc.	0.01 cc.	0.005 cc.	0.0025 cc.	0.001 cc.	0.0005 cc.	0.00025 cc.	0.0001 cc.
		min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
I	Original culture filtrate	*7	8	8	22	36	—	—	—	—	—
II	I, alcohol precipitate, 2.5 mg. per cc.	3	5	8	21	32	—	—	—	—	—
III	I, alcohol precipitate, 10 mg. per cc.	2	2	2	8	16	22	38	—	—	—
IV	I, concentrated and dialyzed	2	2	2	4	4	7	18	27	41	76
V	First elution from adsorbed I	2	2	4	9	15	38	50	—	—	—
VI	Second elution from adsorbed I	2	2	2	5	8	25	34	63	—	—
VII	Third elution from adsorbed I	6	9	9	18	41	—	—	—	—	—
VIII	V, VI, and VII, combined, concentrated	2	2	2	4	5	9	16	23	34	64
IX	VIII, dried and redissolved	2	2	2	4	6	8	19	25	40	55

All tubes incubated in water bath at 37.5°C.

* In Tables I to IV, figures represent time in minutes of interval between clot formation and complete liquefaction; the symbol (—) indicates that no liquefaction occurred in 2 hours.

From the data presented in Table II it is apparent that the fibrinolysin contained in the broth filtrates and in the adsorbed, eluted, and concentrated preparations, is particularly resistant to thermal inactivation. In interesting contrast to this is the fact that the activity of solutions of the alcohol-precipitated material is heat-labile, being partially inactivated after exposure to a temperature of 57°C. for ½ hour, and completely destroyed after 1 hour at this temperature.

TABLE II

Resistance of Fibrinolytic Preparations to Thermal Inactivation

Preparation	Duration of heating	57°C.					75°C.					100°C.				
		0.5 cc.	0.1 cc.	0.05 cc.	0.01 cc.	0.005 cc.	0.5 cc.	0.1 cc.	0.05 cc.	0.01 cc.	0.005 cc.	0.5 cc.	0.1 cc.	0.05 cc.	0.01 cc.	0.005 cc.
		min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
Culture filtrate	1 hr.	3	5	8	19	30	x	8	16	36	40	10	10	16	48	58
Culture filtrate	2 hrs.	3	7	8	18	32	x	x	x	x	x	10	9	15	47	—
Adsorbed, eluted, concentrated	1 hr.	x	1	1	4	8	1	1	4	7	12	4	4	3	10	15
Adsorbed, eluted, concentrated	2 hrs.	x	3	3	4	7	x	x	x	x	x	4	3	3	12	16
Concentrated and dialyzed	1 hr.	x	1	1	4	5	2	2	7	8	16	4	3	8	16	20
Concentrated and dialyzed	2 hrs.	x	1	1	4	7	x	x	x	x	x	4	4	9	20	25
Alcohol precipitated 5 mg. per cc.	15 min.	4	7	—	—	—										
Alcohol precipitated 5 mg. per cc.	30 min.	12	40	—	—	—										
Alcohol precipitated 5 mg. per cc.	45 min.	45	—	—	—	—										
Alcohol precipitated 5 mg. per cc.	1 hr.	—	—	—	—	—										

All tubes in water bath at 37.5°C.

The symbol (x) indicates that the test was not set up.

The reason for the abrupt change in thermal stability following precipitation by alcohol is not clear. The fact that the more potent and, probably, much purer preparations, obtained by the adsorption process, are heat-stable, appears to more closely reflect the correct characterization of the fibrinolysin.

In studies on the effect of heat on the fibrinolysin, different samples of filtrates have not always given results identical with those presented in Table II, especially when higher temperatures were used. Consequently, the experiments were repeated with special consideration being given to the hydrogen ion concentration of the solutions.

Procedure.—Freshly prepared broth was inoculated with streptococci and incubated for 14 hours. The culture was filtered and four 15 cc. portions of the filtrate were adjusted to pH 5.5, 6.5, 7.5, and 8.5, respectively, with accurately measured volumes of N/10 HCl and N/10 NaOH. The tubes were placed in the water bath at 37.5°C. Similar series of preparations were incubated at temperatures of 57°, 75°, and 100°C. (boiling water bath). At intervals of 30 minutes, 1 hour, and 2 hours a test portion of each solution exactly equivalent to 2.5 cc. of the original culture filtrate was withdrawn, cooled rapidly to room temperature, and carefully neutralized with a predetermined volume of N/10 NaOH or N/10 HCl. The test portion was then accurately diluted to a volume of 5 cc. with M/20 phosphate buffer, pH 7.3. Each test solution thus represented a 1 in 2 dilution of the original filtrate. The various solutions were tested at once against the usual rabbit fibrinogen-human thrombin clots. The results of the experiment are presented in Table III.

From Table III it is evident the potency of the culture filtrate is appreciably decreased only upon exposure to temperatures of 75–100°C. At these temperatures the hydrogen ion concentration of the solution is an important factor in the thermal destruction of the fibrinolytic agent. Thus, at pH 5.5 and 8.5 it is seen that the fibrinolytic agent is almost completely destroyed by exposure to a temperature of 100°C. for a period of $\frac{1}{2}$ hour. In solutions at a pH of 6.5 to 7.5, however, the potency of the active substance is decreased only upon longer exposure to this temperature.

Even though the results presented in Tables II and III indicate important factors (method of isolation, and pH) which determine heat stability of fibrinolysin, still other minor discrepancies may be encountered. These latter variants are not yet understood, but seem to be associated with age and composition of the culture medium.

TABLE III

Influence of Hydrogen Ion Concentration of Culture Filtrates on Thermal Inactivation

Duration of heating	pH	37°C.				57°C.				75°C.				100°C.			
		0.25 cc.	0.05 cc.	0.025 cc.	0.01 cc.	0.25 cc.	0.05 cc.	0.025 cc.	0.01 cc.	0.25 cc.	0.05 cc.	0.025 cc.	0.01 cc.	0.25 cc.	0.05 cc.	0.025 cc.	0.01 cc.
		min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
30 min.	5.5	4	5	7	9	4	6	7	11	6	10	20	91?	21	—	—	—
1 hr.	5.5	4	6	7	9	5	9	12	28	7	11	19	50	—	—	—	—
2 hrs.	5.5	4	5	6	10	9	9	13	38	3	11	17	35	—	—	—	—
30 min.	6.5	3	5	15?	18	5	8	9	10	5	8	11	20	5	6	7	26
1 hr.	6.5	5	5	6	11	3	5	7	10	6	8	12	25	7	13	17	60
2 hrs.	6.5	3	4	6	7	4	5	7	11	7	14	20	44	47	—	—	—
30 min.	7.5	4	4	6	8	4	5	6	9	4	5	7	9	6	13	17	38
1 hr.	7.5	4	5	7	9	3	3	6	8	4	6	7	10	10	23	27	—
2 hrs.	7.5	3	5	6	8	4	6	7	9	5	6	10	14	96	—	—	—
30 min.	8.5	4	5	6	8	3	5	6	8	3	5	7	10	10	70	59	—
1 hr.	8.5	4	4	6	7	4	6	8	11	4	8	10	15	—	—	—	—
2 hrs.	8.5	3	5	7	9	4	7	8	11	8	11	19	—	—	—	—	—

All tubes incubated in water bath at 37.5°C.

It may be seen, therefore, that the problem of thermal inactivation is not simple, but the experiments just described serve to characterize the lytic agent as heat-stable.

Effect of Proteolytic Enzymes upon the Fibrinolysin.—In view of the fact that the solutions of the fibrinolysin invariably gave a positive biuret protein test, it was considered of interest to determine the action of proteolytic enzymes upon the active fibrinolytic principle. For this purpose trypsin and papain have been employed.

TABLE IV

Action of Trypsin and Papain upon the Fibrinolysin

Preparation	Duration of incubation	Amount of preparation		
		0.5 cc.	0.1 cc.	0.05 cc.
		min.	*min.*	*min.*
Fibrinolysin + saline (control)	None	3	8	12
Fibrinolysin + saline (control)	30 min.	3	7	10
Fibrinolysin + saline (control)	1 hr.	3	7	11
Fibrinolysin + trypsin	None	4	8	14
Fibrinolysin + trypsin	30 min.	17	—	—
Fibrinolysin + trypsin	1 hr.	—	—	—
Fibrinolysin + papain	None	4	9	12
Fibrinolysin + papain	30 min.	10	25	87
Fibrinolysin + papain	1 hr.	—	—	—

All tubes incubated in water bath at 37.5°C.

Procedure.—Dry, alcohol-precipitated fibrinolytic material was dissolved in M/20 phosphate buffer of pH 7.0. To 4 cc. of this solution was added 1 cc. of a 0.05 per cent solution of commercial trypsin. A similar preparation contained 1 cc. of a 0.05 per cent solution of papain in place of the trypsin. The papain had been previously activated by a trace of HCN. A control solution was prepared by adding 1 cc. of physiological salt solution to 4 cc. of the fibrinolytic solution. The mixtures were incubated at 37.5°C. Portions of each mixture were withdrawn at intervals of 30 minutes and 1 hour and tested in the usual manner. The results of the test are shown in Table IV.

From Table IV it is seen that both trypsin and papain rapidly and completely inactivated the fibrinolysin. This result, considered in connection with the facts that the fibrinolysin is non-dialyzable and is readily adsorbed upon alumina, strongly suggests that the active fibrinolytic principle may be protein in nature. This possibility

corresponds to certain recent developments in enzyme chemistry which have shown that at least three enzymes, urease, pepsin, and trypsin, are proteins (6).

Demonstration of the Fibrinolytic Principle in Reaction Mixtures.—The following experiments were carried out to determine whether or not the fibrinolytic principle is adsorbed by the dissolved fibrin and is demonstrable in the presence of the reaction products.

Procedure. Experiment I.—To 0.2 cc. of rabbit fibrinogen, diluted with 0.2 cc. of physiological saline, 0.5 cc. of a fibrinolytic preparation was added, the activity of which had been shown by titration to be such that 0.0001 cc. caused dissolution, in a usual test in 50 minutes. This mixture was placed in a water bath at 37.5°C. and coagulation was induced by the immediate addition of 0.1 cc. of human thrombin. The time at which coagulation was complete and the interval in minutes between coagulation and liquefaction of the clot were recorded. Immediately after liquefaction of the clot 0.5 cc. of the mixture was withdrawn and added to a tube which contained 0.2 cc. of human fibrinogen and 0.2 cc. of physiological salt solution. Human thrombin, 0.1 cc., was added at once, and the coagulation and liquefaction times again recorded. By successively transferring 0.5 cc. of each digestion mixture to another tube containing fibrinogen, a titration of the active principle in the presence of increasing concentration of dissolved fibrin was accomplished.

In view of certain differences in human and rabbit fibrinogens, to be described in detail later (3), a second and similar titration was carried out in exactly the same manner with the exception that human fibrinogen was substituted for the rabbit fibrinogen. The results of the experiment are shown in Table V.

In Experiment I the test was carried out in such a manner that the fibrinolytic substance was transferred immediately after liquefaction was complete. Under these experimental conditions it may be seen (Table V) that the fibrinolytic agent is not bound as a result of the lytic action. That the fibrinolysin remains, in considerable amount, uninhibited, is evidenced by the fact that the ultimate dilution to 0.001 cc. was effective in 20 minutes. Since the filtrate used in Experiment I, when diluted to 0.0001 cc. with physiological salt solution, effected dissolution in 50 minutes, the observation just described indicates that the fibrinolysin may be present in an active state in the reaction products.

Experiment II.—A mixture of 2.0 cc. of rabbit fibrinogen, 2.0 cc. of physiological saline. and 5.0 cc. of the fibrinolytic solution employed in the above experi-

ment was coagulated by the addition of 1.0 cc. of human thrombin. At stated intervals of incubation 0.5 cc. portions of the liquefied mixture were withdrawn and added to tubes which contained 0.2 cc. of rabbit fibrinogen and 0.2 cc. of salt

TABLE V

Titration of Fibrinolysin Present in the Reaction Mixtures

Tube No.*	Rabbit fibrinogen		Tube No.	Human fibrinogen		Resulting concentration of fibrinolysin
	Coagulation time	Liquefaction time		Coagulation time	Liquefaction time	
	min.	*min.*		*min.*	*min.*	*cc.*
R I	1	2	H I	1	3	0.5000
R II	1	2	H II	2	2	0.2500
R III	1	3	H III	1	3	0.1250
R IV	1	2	H IV	1	4	0.0625
R V	1	2	H V	1	5	0.0318
R VI	1	3	H VI	2	5	0.0159
R VII	1	5	H VII	2	11	0.0079
R VIII	1	8	H VIII	2	30	0.0039
R IX	2	14	H IX	2	25	0.002
R X	2	20	H X	4	—	0.001

All tubes incubated in water bath at 37.5°C.

* Each tube received 0.5 cc. of the contents of the preceding tube; lytic test performed by adding 0.2 cc. fibrinogen, 0.2 cc. of physiological salt solution, and 0.1 cc. of human thrombin. Experiment terminated after tenth transfer.

TABLE VI

Effect of Prolonged Incubation on Activity of the Fibrinolysin in the Presence of the Liquefied Reaction Products

Duration of incubation	Rabbit fibrinogen		Human fibrinogen	
	Coagulation time	Liquefaction time	Coagulation time	Liquefaction time
	min.	*min.*	*min.*	*min.*
15 min.	1	4	1	4
30 min.	1	5	1	4
1 hour	2	5	2	3
2 hours	3	5	2	3
8 hours	4	5	3	3
18 hours	3	5	3	3

All tubes incubated in the water bath at 37.5°C.

solution. Coagulation was induced by the addition of 0.1 cc. of human thrombin. A similar experiment was carried out with human fibrinogen. The results of the experiment are presented in Table VI.

From the table it may be seen that the fibrinolytic activity is not bound by the products resulting from the liquefaction of the fibrin clot and that exposure to the dissolved fibrin for as long as 18 hours exerted only slight inactivating effect upon the fibrinolytic principle. These facts point to the catalytic nature of the active principle, which remains free in test mixtures. Although, in experiments to be described later (3), human and rabbit fibrinogen presented certain differences, in the tests just described no essential dissimilarity was demonstrable.

Optimum Temperature for Fibrinolysis.—The optimum temperature for fibrinolytic action lies between 35° and 45°C. At temperatures of 45–55°C. a partial denaturization of the fibrinogen interferes with the clotting property and above 56°C. actual precipitation occurs. Comparative tests with material kept at ice box temperature, room temperature, and 37.5°C. have been made, and give, on the average, the following results:

In ice box: slight softening, 2 hours.
At room temperature: 10–30 minutes.
At 37.5°C.: 1–2 minutes.

DISCUSSION

The results, which comprise this report, consist of observations which define some of the properties of the fibrinolytic principle elaborated by *beta* hemolytic streptococci.

The active fibrinolysin, present in filtrates of broth cultures, has been isolated by several different procedures. The most successful methods were precipitation with three volumes of alcohol, and adsorption on alumina followed by elution with phosphate buffer. These preparations have been concentrated and evaporated to dryness. The resulting products have retained their potency for indefinite periods of time. Although the active preparations still contain impurities, the isolated lytic material is useful for purposes of standardization and will facilitate further attempts at purification.

The fibrinolysin acts, in many respects, as a relatively stable substance, retaining activity throughout the treatment to which it is subjected and resisting a considerable degree of heating as well as desiccation. Final assay of the properties is, however, rendered difficult because of the fact that the results may vary with different test materials. Some of these conditioning factors have been described; others remain unexplained.

The usual protein tests with preparations of the fibrinolysin are positive and solutions of the active material are rapidly and completely inactivated by the addition of a small quantity of trypsin or activated papain. The methods of isolation and the properties of the active principle strongly suggest that the active agent is an enzyme or catalyst which has a striking specificity for human fibrin and which induces unique physical and chemical changes. Exact chemical identification of the active substance has not yet been accomplished, but all the tests so far performed on impure material point to its protein nature.

SUMMARY

The active fibrinolytic principle present in cultures of hemolytic streptococci can be isolated in stable form, and partially purified by the following methods:

1. Precipitation of culture filtrate with 3 volumes of 95 per cent ethyl alcohol.

2. Adsorption upon polyaluminum hydroxide B of Willstätter (5) followed by elution with M/10 sodium phosphate buffer, pH 7.3.

Concentration can be best accomplished by vacuum dialysis (4) of either culture filtrates or preparations obtained by adsorption and elution.

The streptococcal fibrinolysin is characterized by the following properties:

1. It may resist heating to 100°C. for 60 minutes; variations in thermal resistance are described.

2. Partially purified preparations give positive tests for protein. Activity is rapidly and completely destroyed by trypsin or papain.

3. The active principle is demonstrable in dissolved fibrin even after 18 hours incubation.

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