BIOCHEMICAL STUDIES ON THE FIBRINOLYTIC ACTIVITY OF HEMOLYTIC STREPTOCOCCI

II. Nature of the Reaction

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The preceding paper (1) has described some of the properties of the fibrinolysin of hemolytic streptococci. The present report presents results which indicate the nature of the reaction and the composition of the end-products.

Although the streptococcal fibrinolysin should, in all probability, be classified as an enzyme or catalyst, its activity differs, in certain respects, from proteolytic ferments. The special characters will be demonstrated by comparative experiments in which fibrin and other substrates are subjected to fibrinolysin and other enzymes.

That the fibrinolytic principle of hemolytic streptococci induces a physical change in fibrin is self-evident by the transformation of solid matter to solution. A chemical study of the reaction presents difficulties inherent in attempts to analyze solutions composed of mixtures of proteins. Consequently, the results to be given are not final, but they indicate the qualitative changes which are involved in the phenomenon of fibrinolysis. More exact studies are now in progress and will be reported in a separate communication.

Materials and Methods

The methods of obtaining the fibrinolytic substance from hemolytic streptococci and of preparing fibrinogen and thrombin solutions were described in previous articles (1, 2). It is important to note, however, that human materials have been almost exclusively used in the experiments to be reported. Since the specificity of

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the fibrinolysin for human fibrin is so striking and since this study centers around the reaction products, the necessity of employing human material is apparent.

Where rabbit fibrinogen is employed, special mention of the fact is made.

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

Action of the Fibrinolytic Principle upon Various Substrates

Before proceeding with a description of analytical experiments concerning the effect of fibrinolysin on fibrin, it is desirable to orient the active bacterial substance with respect to other ferments and other substrates. The purpose of each experiment will become apparent from the substrate which was selected and the enzyme which was employed for comparison.

1. Effect of Fibrinolysin and Trypsin on Casein and Gelatin.—These experiments were designed to test the proteolytic properties of the fibrinolytic principle. Casein and gelatin were selected as appropriate substrates, and the experiments were controlled with parallel determinations in which trypsin was substituted for the fibrinolytic principle.

Preparation of Materials.—A 1.25 per cent solution of Bacto gelatin in buffered physiological saline, pH 7.4, was used as a gelatin substrate. A similar solution was prepared from commercial casein which had been purified once by the method of Northrop (3).

The trypsin solution contained 5 mg. of a commercial product per cc. of physiological salt solution.

The fibrinolytic solution was prepared by dissolving 500 mg. of a dry, alcohol-precipitated material in 50 cc. of salt solution. The usual test proved this solution to have a fibrinolytic titer of 0.001 cc. in 34 minutes.

Procedure.—Duplicate 40 cc. portions of the casein substrate were treated with 10 cc. of the trypsin solution and 10 cc. of the fibrinolytic preparation respectively. Two flasks containing 40 cc. of gelatin substrate were similarly subjected to trypsin and fibrinolysin. Control solutions containing one only of each of the various components of the protocol were prepared. Both the test solutions and the controls were incubated at 37.5°C. Toluene was used as a preservative. Analyses were carried out at once and after 24 and 48 hours incubation.

Analytical Method.—A 5 cc. portion of each digestion mixture was used for each analysis. The accurately measured sample was placed in the deaminizing bulb of a Van Slyke *macro* amino nitrogen apparatus and deaminization was permitted to proceed for 15 minutes. The evolved nitrogen was collected and measured in a micro burette. Duplicate determinations were made upon each solution and the

values usually agreed within 0.02 ml. of evolved nitrogen. The results of the analyses, appropriately corrected for the blank error and the amino nitrogen content of the trypsin and fibrinolytic solutions, are presented in Table I and in Text-fig. 1.

From Table I and Text-fig. 1, it is evident that, by the analytical method used, the fibrinolytic substance exerted no demonstrable hydrolytic action upon either of the two proteins, casein or gelatin. The possibility of a superficial hydrolysis, not detectable by the analytical method employed, seems improbable from the fact that a 2 per

TABLE I

Comparison of the Action of Fibrinolysin and Trypsin upon Casein and Gelatin

Preparation	Duration of incubation	Amino N per 100 cc.*	
		Found	Increase
		mg.	*mg.*
Casein + trypsin	None	3.27	—
Casein + trypsin	24 hours	15.10	11.83
Casein + trypsin	48 hours	17.04	13.77
Casein + fibrinolysin	None	3.24	—
Casein + fibrinolysin	24 hours	3.20	−0.04
Casein + fibrinolysin	48 hours	3.15	−0.09
Gelatin + trypsin	None	3.52	—
Gelatin + trypsin	24 hours	19.19	15.67
Gelatin + trypsin	48 hours	21.71	18.19
Gelatin + fibrinolysin	None	3.52	—
Gelatin + fibrinolysin	24 hours	3.67	0.15
Gelatin + fibrinolysin	48 hours	3.79	0.27

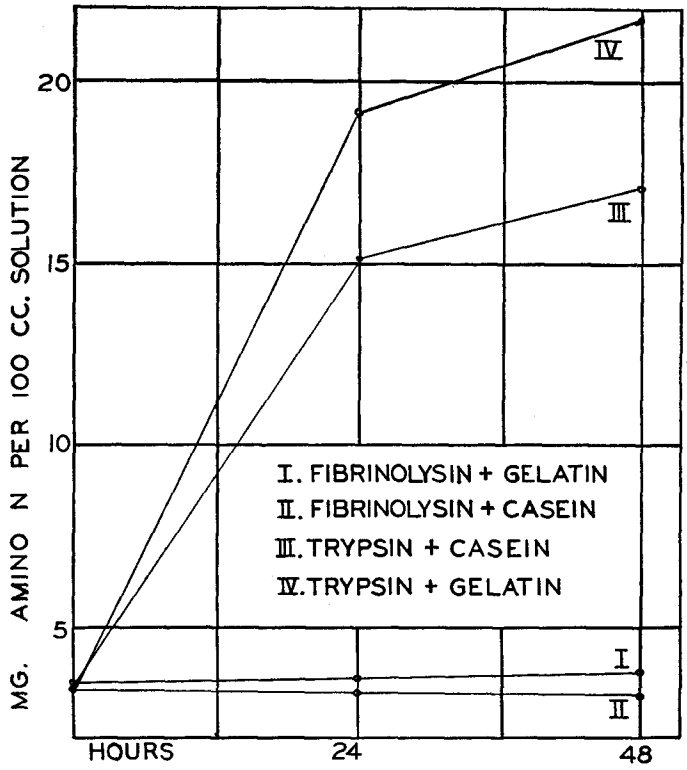
* All values corrected for blank error and for amino nitrogen content of the trypsin and fibrinolysin solutions, respectively.

cent gelatin solution which contained 10 mg. of the fibrinolytic substance per cc. promptly solidified when it was placed in an ice bath after incubation for 72 hours at 37.5°C.

2. Effect of Fibrinolysin and Peptase (Stevens and West) on Peptone.— Stevens and West (4) have reported the presence of a proteolytic enzyme in the cell extracts of hemolytic streptococci. This enzyme was shown to attack casein, but maximal hydrolysis was observed when a peptone substrate was employed. The term peptase was therefore applied to the enzyme. The following experiment was designed to

demonstrate the difference between the fibrinolytic substance and the streptococcus peptase of Stevens and West.

Preparation of Materials. Peptase.—The sedimented cells from 3 liters of broth culture were thoroughly washed with physiological salt solution and suspended in 5 cc. of sterile distilled water. The suspension was frozen and subjected to pres-



TEXT-FIG. 1. Comparison of the action of fibrinolysin and trypsin upon casein and gelatin.

sure in the apparatus described by Johlin and Avery (5).[1] The resulting mixture was warmed to room temperature, diluted to a volume of 15 cc. with physiological saline, and shaken for 15 minutes to insure complete emulsification. The solution was then centrifuged at a high speed for an hour. The supernatant fluid was

[1] The authors are indebted to Dr. R. C. Avery of the Department of Bacteriology, Vanderbilt University, who kindly supplied the extraction apparatus.

reserved and the sediment was again frozen and subjected to disintegration by pressure. The mixture was diluted, shaken, and again centrifuged at a high speed. By microscopical examination of stained smears, most of the bacterial cells were found to be ruptured. The combined extracts, 25 cc. in volume, were mixed with a little toluene and permitted to stand in the ice box for 12 hours. The solution was divided into two parts, one of which was heated at 62°C. for 1 hour.

The fibrinolytic preparation used for comparison with the peptase was prepared by the method of adsorption and elution, just described. The eluate titred to 0.001 cc. in 14 minutes. A portion of the preparation was heated at 62°C. for 1 hour.

Procedure.—To four 10 cc. portions of a 1 per cent solution of Merck peptone were added respectively 5 cc. of the peptase solution (cell extract), 5 cc. of the fibrinolytic solution, 5 cc. of the heated peptase, and 5 cc. of the heated fibrinolytic preparation. Appropriate controls of the peptone and other solutions were

TABLE II *a*

Comparison of the Action of Fibrinolysin and Streptococcus Peptase upon Peptone

Preparation	Amino N per 100 cc.*	
	Found	Increase
	mg.	*mg.*
Peptone alone	28.82	—
Peptone + fibrinolysin	28.87	0.05
Peptone + heated fibrinolysin	28.85	0.03
Peptone + streptococcus peptase	44.36	15.54
Peptone + heated streptococcus peptase	28.65	−0.17

* All values corrected for blank error and for amino nitrogen content of fibrinolysin and peptase solutions, respectively.

prepared. A portion was withdrawn from each tube for immediate analysis. The remainder of the solutions were layered with a little toluene and incubated at 37.5°C. Samples were withdrawn for analysis after 24 to 48 hours incubation.

Analytical Method.—Analyses were carried out upon 2 cc. portions of each solution. The samples were deaminized for 15 minutes in the *micro* Van Slyke amino nitrogen apparatus and the evolved nitrogen collected and measured in the micro burette. Duplicate determinations upon each solution agreed within 0.02 ml. of evolved nitrogen. The results of the experiment are shown in Table II *a*.

From Table II *a* it is evident that the fibrinolytic principle is not capable of hydrolyzing peptone, whereas the cell extract exerted an intensive peptolytic action upon the substrate. It is also evident from the table that heating the peptase at 62°C. for 1 hour destroyed its enzymatic action for peptone.

A comparison of the fibrinolytic titers of the fibrinolysin and peptase solutions was carried out and the results are recorded in Table II *b*.

From Table II *b* it can be seen that the fibrinolytic solution possessed a uniformly high titer which was unaltered by exposure to a temperature of 62°C. The cell extract, on the other hand, contained only a comparatively small amount of fibrinolytic active material which probably represents the amount of intracellular fibrinolysin. The fact that exposure to a temperature of 62°C. for an hour diminished the small amount of fibrinolytic activity in the cell extract may have been

TABLE II *b*

Comparison of the Fibrinolytic Action of Fibrinolysin and Streptococcus Peptase

Preparation	Amount of preparation					
	0.5 cc.	0.1 cc.	0.05 cc.	0.01 cc.	0.005 cc.	0.001 cc.
	min.	*min.*	*min.*	*min.*	*min.*	*min.*
Fibrinolysin	*3	3	6	10	12	14
Heated fibrinolysin	3	3	7	14	14	14
Streptococcus peptase	36	24	20	35	70	—
Heated streptococcus peptase	120	120	90	—	—	—

All tubes incubated in water bath at 37.5°C.

* Figures represent time in minutes of interval between coagulation and complete liquefaction; the symbol (−) indicates that no liquefaction occurred in 2 hours.

due to an adsorption of the fibrinolytic principle upon the extraneous heat-denatured protein material in the heated extract.

The results of the experiment are considered to justify the conclusion that the fibrinolytic activity of streptococci is referable to an active substance, chemically distinct from the peptolytic enzyme previously isolated from streptococci by Stevens and West.

3. Effect of Fibrinolysin, Trypsin, and Peptase on Fibrin.—The negative character of the above experiments indicates that the fibrinolytic principle does not possess a broad capacity to act upon proteins in general or upon peptone. That fibrin is affected is self-evident from the physical change which the clot undergoes. The nature of the chemical change in fibrin is indicated in experiments to follow. In

order to bring out the special qualities of the fibrinolysin, trypsin and streptococcal peptase have been employed in comparable experiments.

Preparation of the Fibrin.—Potassium oxalate was employed as an anticoagulant. Human plasma was diluted with five times its volume of physiological salt solution and coagulation was induced by the addition of an excess of $CaCl_2$. The coagulum was permitted to stand several hours to insure complete formation of the fibrin. By careful manipulation of a stirring rod the liquid was pressed from the gel and the fibrin rolled into a compact mass. This product was washed thoroughly with distilled water, pressed dry, and placed in a vacuum desiccator over phosphoric anhydride. The resulting brittle product was ground to a fine white

TABLE III

Comparison of the Action of Fibrinolysin, Streptococcus Peptase, and Trypsin upon Human Fibrin

Preparation	Amino nitrogen per 100 cc.*			
	24 hrs.	Increase	72 hrs.	Total increase
	mg.	*mg.*	*mg.*	*mg.*
Fibrin alone	7.37	—	7.89	0.52
Fibrin + fibrinolysin	13.18	5.81	18.33	10.96
Fibrin + heated fibrinolysin†	8.57	1.20	9.55	2.18
Fibrin + streptococcus peptase	26.40	19.03	25.78	18.41
Fibrin + heated streptococcus peptase	9.50	2.13	9.98	2.61
Fibrin + trypsin	37.79	30.22	43.83	36.46

* All values corrected for blank error and for the amino nitrogen content of fibrinolysin, streptococcus peptase, and trypsin solutions, respectively.

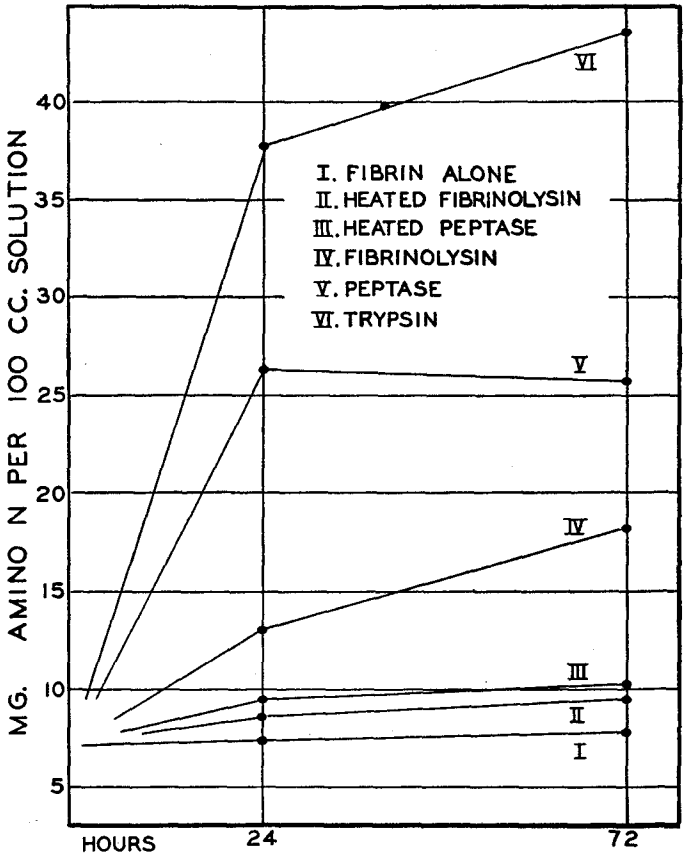
† The fibrinolysin employed in this experiment had been precipitated with alcohol and was therefore heat-labile.

powder in a mortar. The combined and uniformly mixed fibrin from several preparations was utilized in the following experiment.

Active Solutions.—A solution of commercial trypsin was made up in a concentration of 4 mg. per cc. of physiological salt solution. A streptococcal peptase solution was prepared in the manner just described. The peptase solution, 25 cc. in volume, contained the material extracted from the sedimented cells from 4 liters of broth culture. The solution was divided and one portion was heated at 60°C. for 1 hour. The fibrinolytic solution contained 10 mg. of an alcohol-precipitated preparation per cc. of salt solution. The titer of this solution was 0.001 cc. in 43 minutes. A portion of the solution was heated for an hour at 60°C.

Procedure.—Duplicate series of small flasks were prepared, each of which con-

tained 200 mg. of the powdered fibrin and 15 cc. of buffered saline, pH 7.2. Accurately measured 5 cc. portions of the various test solutions were added to the substrate suspensions, and the mixtures, together with appropriate controls, were placed in the incubator at 37.5°C. Toluene, 1 cc., was added to each flask to insure sterility. One group of flasks was removed after 24 hours incubation, the con-



TEXT-FIG. 2. Comparison of the action of fibrinolysin, streptococcus peptase, and trypsin upon human fibrin.

tents carefully filtered, and amino nitrogen determinations carried out upon aliquot portions of the filtrate. The second series of flasks were removed from the incubator after 72 hours and the contents similarly treated. The details of the analytical method were the same as those described in the experiments with casein and gelatin. The results of these analyses are presented in Table III and in Text-fig. 2.

The values recorded in Table III and Text-fig. 2 present an interesting contrast to the results of experiments with the casein, gelatin, and peptone substrates. Trypsin hydrolyzed the fibrin with a sharp increase in the amino N according to the well recognized proteolytic action of this ferment. The fact that the peptase possesses the property of hydrolyzing fibrin is of interest in view of the results of Stevens and West who found this enzyme incapable of hydrolyzing either serum or serum albumin.

Of more immediate interest to the present purpose, however, is the fact that, at the end of 72 hours incubation, a not inconsiderable amount of amino nitrogen was demonstrated to be present in the fibrin-fibrinolytic principle mixture. A correct evaluation of this increase is not yet possible. Two interpretations may be considered. The results may signify that the fibrinolytic principle is comparable to a proteolytic enzyme which acts specifically in promoting the hydrolysis of human fibrin. On the other hand, a more probable explanation lies in the dissolving action of the fibrinolytic substance upon the insoluble fibrin substrate. Under this condition the increase in the amino nitrogen may be accounted for by an increased protein concentration of the solution. From this standpoint it is perhaps significant that the increase in amino nitrogen in the mixtures took place at a more gradual and constant rate than was observed with either trypsin or the peptase. Furthermore, this latter view, which implies that the chemical structure of fibrin is not extensively altered by the process of dissolution, receives additional support from the fact that the end-product of the reaction acts, in other tests, as a protein. A study of the mechanism of the reaction is being continued by experimental procedures which, it is hoped, will leave no question with regard to the rôle of hydrolysis in the dissolution of the fibrin.

4. Effect of Fibrinolysin on Fibrinogen.—In this and previous reports emphasis has been placed upon the fact that the fibrinolytic principle acts specifically upon clotted human fibrin and fails to act on rabbit fibrin when composed entirely of rabbit constituents. It became of interest to determine the activity of fibrinolysin upon the two species of fibrinogens, precursors of fibrin. The technique of the experiments and the results are as follows.

Procedure.—Human fibrinogen, 4 cc., was diluted with 4 cc. of physiological salt solution. A 10 cc. portion of an adsorbed, eluted, and concentrated fibrinolytic preparation was added and the mixture placed in the 37.5°C. water bath. A similar solution containing rabbit fibrinogen and the fibrinolytic solution was prepared and incubated simultaneously. At frequent intervals, during incubation, 0.9 cc. samples of each solution were withdrawn, and mixed with 0.1 cc. portions of human thrombin. The coagulation time and the interval required for liquefaction of the clots were recorded. The results are shown in Table IV.

The table shows that when fibrinolysin and human fibrinogen are incubated for 20 minutes, the clotting power of the fibrinogen is seri-

TABLE IV

Comparison of the Action of Fibrinolysin upon Human and Rabbit Fibrinogen

Duration of incubation of fibrinogen with fibrinolysin	Human fibrinogen		Rabbit fibrinogen	
	Coagulation time	Liquefaction time	Coagulation time	Liquefaction time
	min.	*min.*	*min.*	*min.*
None	*1	7	1	6
5 minutes	2	6	2	4
10 minutes	2	5	2	4
20 minutes	†2	5	1	4
35 minutes	—	—	1	4
50 minutes	—	—	2	3
5 hours	—	—	2	4
18 hours	—	—	2	5

All tubes incubated in water bath at 37.5°C.

* Figures represent time in minutes; the symbol (—) indicates no coagulation occurred in 2 hours.

† Clot imperfectly formed.

ously impaired, and that after 35 minutes incubation, the fibrinogen no longer is capable of forming fibrin clot. In contrast to the change induced in the human protein, rabbit fibrinogen, even after 18 hours exposure to fibrinolysin, remains unchanged and promptly solidifies when thrombin is added. This resistance of rabbit fibrinogen presents an interesting biochemical difference between the two species of fibrinogens and is undoubtedly an important factor in determining the insusceptibility of rabbit fibrin to the fibrinolytic action.

The Protein Nature of the Reaction Products

The experiments to be described consist of a comparison of the well defined properties of fibrinogen solutions with those of solutions containing the products of fibrinolysis and solutions of fibrinogen which have been exposed to and altered by fibrinolysin.

Human fibrinogen is a salt-soluble, water-insoluble globulin, precipitable by 50 per cent saturation with sodium chloride, or 25 per cent saturation with ammonium sulfate. It is denatured by exposure to a temperature of 57°C. It is particularly characterized by its transformation into the insoluble fibrin through the agency of thrombin.

A study of these four properties of fibrinogen have, therefore, been applied to the products of fibrinolysis and to fibrinogen-fibrinolysin mixtures. In these experiments, materials derived from human sources have been exclusively employed.

1. Precipitation with NaCl.—When NaCl is added up to 50 per cent saturation to the products of fibrinolysis no precipitate or clouding occurs. Similarly after fibrinogen-fibrinolysin mixtures have been incubated for 60 minutes, 50 per cent NaCl saturation exerts no precipitating effect.

2. Precipitation with $(NH_4)_2SO_4$.—When $(NH_4)_2SO_4$ is added to the products of fibrinolysis in amounts sufficient for 25 per cent saturation, no precipitation occurs. The same negative result is obtained with incubated fibrinogen-fibrinolysin mixtures.

When, however, in the products of fibrinolysis or fibrinogen-fibrinolysin mixtures the saturation of $(NH_4)_2SO_4$ is raised to 35 per cent, a large yield of precipitate is obtained. Furthermore, by increasing the saturation to 40 and 45 per cent additional flocculation occurs. These results are of interest since the fibrin after dissolution, and the fibrinogen-fibrinolysin mixtures after incubation, are abundantly precipitated by the per cent saturations of $(NH_4)_2SO_4$ which flocculate serum globulins.

3. Effect of Heat.—Fibrinogen, the products of fibrinolysis, and fibrinogen-fibrinolysin mixtures were incubated at 37.5°C. for 1 hour. Each preparation was then heated at 57°C. for 30 minutes. The untreated fibrinogen flocculated abundantly; in the other two preparations, only a moderate cloud and a slight precipitation occurred. Heating the latter two solutions at 75°C. caused a heavy precipitation. Quantitative studies are now in progress to define more exactly the differences demonstrable in these experiments with different temperatures.

4. Addition of Thrombin.—Untreated fibrinogen, the products of fibrinolysis, and fibrinogen-fibrinolysin mixtures were incubated at 37.5°C. At intervals of 5, 10, 15, and 30 minutes, samples of each were removed and mixed with thrombin. With the fibrinogen, clotting took place promptly with each specimen. With

the products of fibrinolysis, the addition of thrombin was entirely without effect. With the fibrinogen-fibrinolysin mixture, clotting occurred only in the 5 minute samples; in the 10 minute sample a few strands of fibrin appeared; in the remaining test no coagulation occurred.

The qualitative character of the products resulting from the action of streptococcal fibrinolysin upon fibrin and fibrinogen is shown by the experiments just described. The results indicate that, although the changes are distinct, the end-product still has properties of protein, which is apparently newly formed. The results further substantiate the opinion that the dissolution of the fibrin is not accompanied by extensive hydrolysis.

Investigations of a more exact identification of the end-product are now in progress.

DISCUSSION

A comparison of the action of streptococcal fibrinolysin and other enzymes on fibrin and other substrates, demonstrates certain unique biochemical properties of the bacterial product.

The specificity of the fibrinolysin is particularly interesting. Differences in action on human and animal clot have been previously reported (1, 6). The insusceptibility of rabbit plasma clot finds a probable explanation in the fact that rabbit fibrinogen is not altered by the fibrinolysin. The basis of the resistance of rabbit material in contrast to the susceptibility of human protein seems to depend upon a difference in the chemical constitution of the two substances even though they both are physiologically precursors of fibrin.

Additional evidence of the specificity is brought out by the apparent incapacity of the fibrinolysin to attack casein, gelatin, or peptone. The fibrinolytic principle does not act upon proteins in general and its limitations are strikingly exemplified by contrast to trypsin. Of equal interest is the sharp difference between peptase and fibrinolysin both derived from hemolytic streptococci. The peptase of Stevens and West is an intracellular proteolytic enzyme especially potent against peptone. The fibrinolysin is a product of the same cultures but is chiefly extracellular and possesses different capacities.

At the present time the experimental data have not clearly defined the course of the fibrinolytic reaction. Whether or not the dissolution

is accompanied by definite proteolytic hydrolysis is uncertain, and awaits further study. However, the fact that the end-products, even after 72 hours incubation, still have the characteristics of a protein, indicates the unusual effect exerted by the streptococcal fibrinolysin. The gradual and constant increase in the amino nitrogen content of a mixture containing the fibrinolytic principle and human fibrin suggests that, in addition to the dissolution of the insoluble substrate, an unexplained and perhaps very slight splitting of the molecule has taken place. It would be premature, at the present time, to speculate concerning the correct interpretation. Studies now in progress will attempt to define more accurately the mechanism involved in the fibrinolytic phenomenon.

SUMMARY

The fibrinolysin of hemolytic streptococci exerts no hydrolytic action upon casein, gelatin, or peptone.

The action on solid human fibrin is characterized by a small and gradual increase in the amino nitrogen content of the solution.

The specific and special enzymatic action of fibrinolysin is contrasted with trypsin and with streptococcal peptase (4).

Solutions of human fibrinogen, after brief incubation with fibrinolysin, lose the capacity to form fibrin. Solutions of rabbit fibrinogen, on the other hand, retain the property of transformation into fibrin, even after prolonged exposure to fibrinolysin.

Qualitative tests, with solutions resulting from the action of streptococcal fibrinolysin on human fibrin, indicate that the end-product may be protein and that the degradation of the molecule is not great.

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