STUDIES ON A PROTEOLYTIC ENZYME IN HUMAN PLASMA

II. Some Factors Influencing the Enzymes Activated by Chloroform and by Streptococcal Fibrinolysin*

By OSCAR D. RATNOFF, M.D.

(From the Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore)

(Received for publication, November 14, 1947)

Mammalian plasma contains a proteolytic enzyme in inactive form. Early studies indicated that in shed blood the enzyme gradually became activated spontaneously, and the blood acquired proteolytic properties (1, 2). When the plasma was treated with a variety of substances, including chloroform or other organic solvents (3) its proteolytic activity was greatly increased, although the activation proceeded slowly. Filtrates of cultures of beta hemolytic streptococci also increased the proteolytic activity of plasma (4), apparently by activating the same proteolytic enzyme as chloroform (2, 5, 6). Activation of plasma proteolytic enzyme with streptococcal fibrinolysin, however, is dramatic and sudden, suggesting that the mechanism of action of chloroform and streptococcal fibrinolysin differ.

In order to delineate the conditions necessary for the quantitative determination of plasma proteolytic enzyme, a study has been made of the activation of the enzyme by chloroform and by streptococcal filtrates. This study indicates that activation of plasma proteolytic enzyme by chloroform is incomplete and slow, requiring at least 16 hours for the development of maximal activity. On the other hand, activation with maximal amounts of streptococcal fibrinolysin occurs almost instantly.

Earlier reports (5, 7) suggested that the activation of plasma proteolytic enzyme by streptococcal fibrinolysin is a catalytic process. Data will be presented in this paper which indicate that in the presence of some substance altered by chloroform or heat, the activation of plasma proteolytic enzyme by streptococcal fibrinolysin behaved as if it involved a stoichiometric reaction. Streptococcal fibrinolysin seemed to react in molecular proportions with a substance in plasma euglobulin which limited the activation of plasma proteolytic enzyme. These data reopen the question as to the mode of activation of plasma proteolytic enzyme by streptococcal fibrinolysin.

The quantitative estimation of enzymatic activity requires a knowledge of the influence of changes in concentration of enzyme and substrate upon the course of digestion. Earlier studies (8) indicated that the digestion of gelatin

* This work was supported by a grant from the United States Navy Office of Research and Inventions.

by plasma proteolytic enzyme was proportional to the square root of the concentration of enzyme, and to the square root of the duration of digestion. That is, plasma proteolytic enzyme was said to obey Schütz's law.

The rate of digestion by plasma proteolytic enzyme has been reinvestigated. These studies suggest that like other hydrolytic enzymes, plasma proteolytic enzyme digests casein in direct proportion to the concentration of the enzyme and to time, at least during the early course of digestion. Furthermore, it has been shown that the plasma proteolytic enzyme preparation, when activated by filtrates of cultures of beta hemolytic streptococci, deteriorates after incubation for approximately 1 hour; but the same enzyme activated by chloroform does not deteriorate for several hours. These studies indicate that chloroform may remove some substance from plasma which permits plasma proteolytic enzyme to deteriorate.

Methods

The nomenclature and all the methods employed in the following experiments have been described in the preceeding paper (6).

EXPERIMENTAL

I. The Kinetics of Activation of a Plasma Proteolytic Enzyme by Chloroform

1. The Optimal Duration and Temperature of Incubation of Plasma Globulin and Chloroform in the Activation of Plasma Proteolytic Enzyme.—The quantitative determination of the amount of chloroform-activated plasma proteolytic enzyme requires a knowledge of the optimal duration of incubation of the chloroform-globulin mixture, and the optimal temperature of this activation. When chloroform is added to whole plasma, the proteolytic activity of the mixture slowly increases, reaches a maximum in 7 to 10 days, and then decreases (3, 9). The following experiments indicate that the peak of proteolytic activity is reached in a much shorter time if the precursor of plasma proteolytic enzyme is first separated from the rest of the plasma by precipitation with the euglobulin fraction at pH 5.2.

In order to determine the optimal time of activation of plasma proteolytic enzyme with chloroform, equal amounts of chloroform were added to aliquots of a solution of plasma euglobulin in buffer, and the resultant mixtures then incubated. The course of activation was determined by measuring the proteolytic activity of aliquots which were incubated for increasingly long periods of time.

In a typical experiment, 20 cc. of plasma was added to 380 cc. of distilled water, and sufficient 1 per cent acetic acid was added to bring the hydrogen ion concentration to pH 5.2. The resultant precipitate was separated by centrifugation and dissolved in 22 cc. of buffer. One cc. portions of the globulin solution were pipetted into each of 21 large test tubes, and half a cc. of chloroform was added to each. The tubes were then stoppered and shaken for

10 seconds. Ten tubes were placed in a water bath at 25°C. and 10 in a bath at 37°C. The proteolytic activity of the twenty-first mixture was determined immediately. After appropriate intervals, the activity of the other mixtures at each temperature was measured. To determine proteolytic activity, the contents of each tube were transferred to a graduated centrifuge tube and the aqueous phase diluted with buffer to 5 cc. The chloroform and precipitate were separated by centrifugation. Two cc. of 0.3 per cent casein in 0.004 N sodium hydroxide was then added to a 2 cc. aliquot of chloroform-activated enzyme, and the amount of casein digested in 1 hour at 37°C. was determined nephelometrically.

As can be seen in Fig. 1 short periods of incubation of the chloroform-globulin mixture were unsatisfactory, because the amount of proteolytic activity present

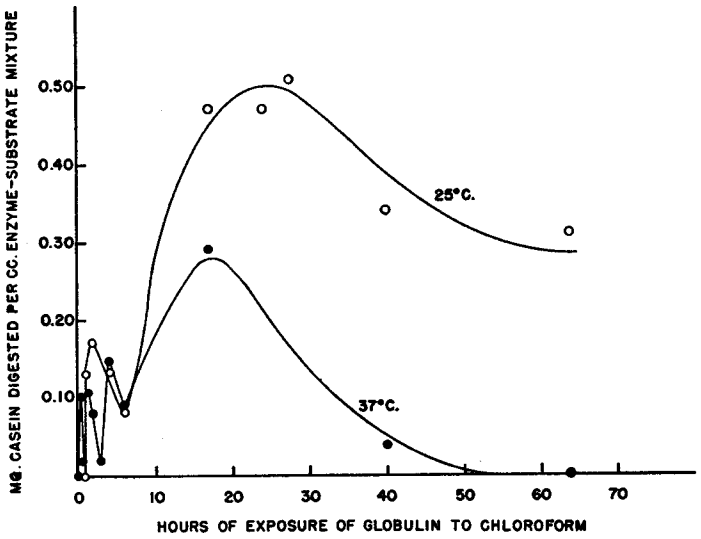

FIG. 1. The influence of the duration of incubation of chloroform-globulin mixtures on plasma proteolytic activity at 25° and 37°C. See text.

was extremely variable. This may have been due to the formation of a pale gray clot which could be broken up only with difficulty. After about 8 hours of incubation the clot lysed and thereafter consistent results were obtainable. The peak of activity was reached between 16 and 28 hours, after which activity declined.

Incubation of the chloroform-globulin mixture at 25°C. resulted in much greater proteolytic activity than incubation at 37°C. No measurable proteolytic activity was present after incubation of the mixture at 4° or 56°C. for 17 hours. These experiments indicate that incubation of a mixture of plasma euglobulin solution and chloroform for 16 to 28 hours at 25°C. was the most effective of the combinations of time and temperature tested in the activation of plasma proteolytic enzyme.

2. The Optimal Values of Buffer and Chloroform Necessary for the Maximal Activation of the Proteolytic Enzyme in 2 cc. of Plasma.—The quantity of buffer in which the euglobulin precipitate of plasma was dissolved had a material effect upon the proteolytic activity which appeared upon incubation with chloroform. If the globulin precipitate was dissolved in more than 2 cc. of buffer before the addition of chloroform, very little active enzyme was found. Table I tabulates the results of an experiment designed to demonstrate which volume of buffer, used to dissolve the euglobulin precipitate of 2 cc. of plasma,

TABLE I

Influence on Proteolytic Activity Evoked by 0.5 Cc. of Chloroform of the Volume of Buffer Used to Dissolve the Euglobulin Precipitate of 2 Cc. of Plasma

Volume of buffer	Enzymatic activity
cc.	*mg./cc.**
0.5	0.70
1.0	0.68
2.0	0.44

* Milligrams of casein digested per cubic centimeter of enzyme-substrate mixture in 1 hour.

TABLE II

Influence on Proteolytic Activity of the Volume of Chloroform Added to the Euglobulin Precipitate of 2 Cc. of Plasma Dissolved in 0.5 Cc. of Buffer

Volume of chloroform	Enzymatic activity
cc.	*mg./cc.**
0.1	0.43
0.3	0.43
0.5	0.46
1.0	0.42

* Milligrams of casein digested per cubic centimeter of enzyme-substrate mixture in 1 hour.

resulted in greatest enzymatic activity. As can be seen, the maximal proteolytic activity observed was in the tubes in which the globulin precipitate of 2 cc. of plasma was dissolved in 0.5 cc. of buffer. This volume was used in most experiments, although in some the globulin was dissolved in 1.0 cc. of buffer.

The volume of chloroform added to the buffered globulin solution was of importance, although this was minimized by thorough mixing before incubation. In experiments in which chloroform and globulin were mixed thoroughly, a volume of 0.5 cc. of chloroform evoked the most proteolytic activity when the globulin was dissolved in a volume of 0.5 cc. or 1.0 cc. (Table II). In some experiments in which mixing was not thorough, a volume of chloroform of 0.2 cc. or less induced much less activity than 0.5 cc. Furthermore, repeated experiments indicated that a volume of chloroform of 1.0 cc. resulted in less

activity than 0.5 cc., perhaps because the larger volume of chloroform denatured more of the enzyme.

In summary, the proteolytic activity evoked by chloroform was maximal under the conditions studied when the euglobulin precipitate of 2 cc. of plasma was dissolved in 0.5 cc. of buffer, mixed thoroughly with 0.5 cc.o f chloroform, and incubated for 16 to 28 hours at 25°C.

II. The Kinetics of Activation of Plasma Proteolytic Enzyme by Streptococcal Fibrinolysin

1. The Influence of Increasing Concentrations of Streptococcal Fibrinolysin upon the Immediate Proteolytic Activity of Plasma Globulin.—In order to determine the amount of proteolytic enzyme in plasma which can be activated by streptococcal fibrinolysin, the effect of increasing concentrations of streptococcal fibrinolysin upon proteolytic activity was studied.

In a typical experiment, the euglobulin precipitate of 16 cc. of pooled plasma was dissolved in 15 cc. of buffer. Varying volumes of fibrinolysin prepared from the H46A strain of beta hemolytic streptococcus were then added to 1 cc. aliquots of the globulin solution, and the volume in each tube brought to 5 cc. with buffer. One cc. aliquots of these mixtures were immediately diluted with 1 cc. of buffer, and 2 cc. of 0.3 per cent casein in 0.006 N sodium hydroxide was added. The progress of digestion during 1 hour at 37°C. was measured.

As may be seen in the upper curve of Fig. 2, the proteolytic activity of the globulin solution increased as the concentration of fibrinolysin increased, until a maximum was reached.

2. The Stability of Streptococcal Fibrinolysin-Activated Enzyme.—Christensen (5) demonstrated that the plasma proteolytic enzyme activated by small amounts of fibrinolysin did not reach the peak of its activity for several hours. The experiments to be described suggest, on the contrary, that prolonged incubation of small amounts of fibrinolysin with a solution of globulin which has otherwise been untreated does not increase proteolytic activity. Indeed, the activity of globulin activated by fibrinolysin began to decrease within the 2nd hour, regardless of the concentration of fibrinolysin.

In the experiment described in the preceeding section, 1 cc. aliquots of the globulin-fibrinolysin mixtures were incubated for 4 hours at 37°C. before the addition of casein, and the proteolytic activity of the mixture was then measured as before. The proteolytic activity of another set of 1 cc. portions of the same globulin-fibrinolysin mixtures was then measured after incubation for 3½ hours at 25°C.

As can be seen in Fig. 2, the fibrinolysin-induced proteolytic activity of globulin had decreased after incubation for 3½ hours. The decrease was apparently greater at 37°C. than at 25°C., and as marked with low as with high concentrations of fibrinolysin. That is, after the proteolytic enzyme in plasma euglobulin had been activated by streptococcal fibrinolysin, its activity decreased rapidly.

3. The Interrelation of the Concentrations of Streptococcal Fibrinolysin and Plasma Globulin, and Plasma Proteolytic Activity.—The activation of plasma proteolytic enzyme by streptococcal filtrates has been said to be catalytic in nature (5, 7). The hyperbolic relationship between fibrinolysin concentration and proteolytic activity described above might be explained otherwise, namely, that streptococcal filtrates may activate plasma proteolytic enzyme by reaction with some substance in plasma in accordance with the law of mass action. If this were true, then the amount of proteolytic activity evoked by mixtures of

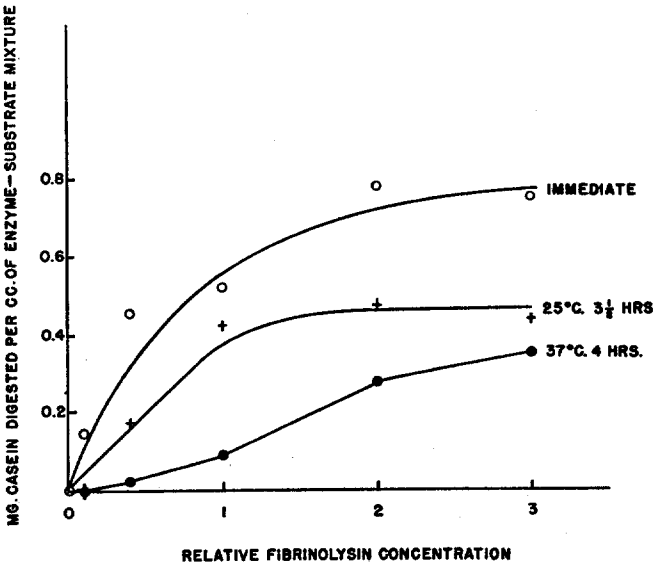

FIG. 2. The influence of increasing concentrations of streptococcal fibrinolysin on the proteolytic activity of plasma globulin solution, and the effect of incubation on the activated emzyme. Proteolytic activity is plotted against the amount of fibrinolysin added to globulin solution. The upper curve represents the proteolytic activity immediately after the addition of fibrinolysin; and the middle and lower curves, the proteolytic activity after the fibrinolysin-globulin mixtures had been allowed to stand at 25 and 37°C. for 3½ and 4 hours respectively.

streptococcal filtrates and of globulin should depend upon the product of their respective concentrations. The following experiment was designed to test this hypothesis.

A series of dilutions of streptococcal fibrinolysin was prepared, using buffer as a diluent. Similarly, plasma globulin was prepared in two dilutions, using buffer as a diluent. Equal volumes of each dilution of streptococcal fibrinolysin and of each dilution of globulin were mixed. This resulted in a series of tubes in which the fibrinolysin and globulin concentrations varied, but the products of the concentrations of fibrinolysin and globulin coincided at various points. To each mixture an equal volume of 0.3 per cent casein was added, and the enzyme-substrate mixture was then incubated at 37°C. for 1 hour.

The data from one experiment such as this have been plotted in Fig. 3. The ordinate represents the enzymatic activity of each preparation, expressed in milligrams of casein digested during the 1 hour period. The abscissa represents the product of the concentrations of fibrinolysin and globulin, expressed arbitrarily. As can be seen, the effect on enzymatic activity of doubling the concentration of fibrinolysin is the same as the effect of doubling the concentration of globulin within the limits tested. These results are compatible with a stoichiometric reaction.

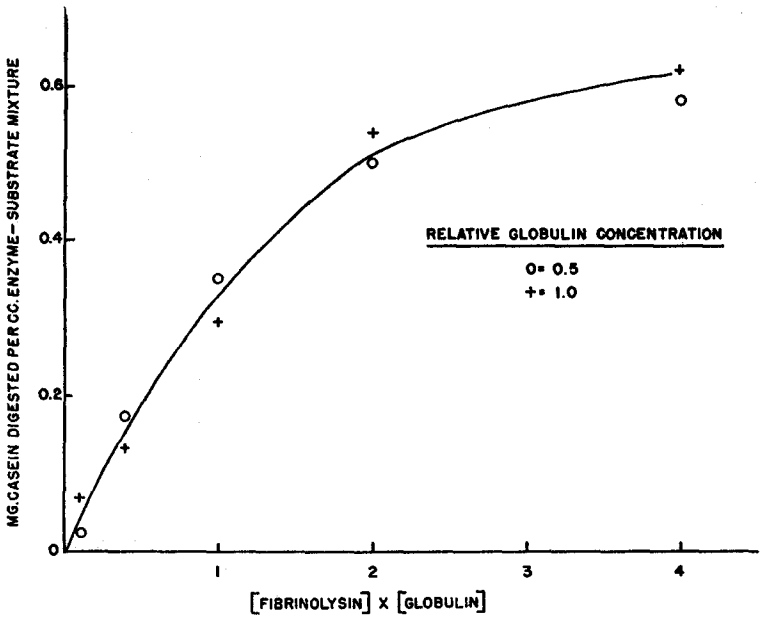

FIG. 3. The relationship between the product of the concentrations of fibrinolysin and globulin solutions and plasma proteolytic activity. The concentrations of fibrinolysin and globulin were varied independently and the resultant proteolytic activity was plotted against the product of the concentrations of fibrinolysin and globulin. See text.

4. The Effect of Fresh Fibrinolysin on Deteriorated Fibrinolysin-Activated Enzyme.—The experiments just described suggested that when streptococcal fibrinolysin activates plasma proteolytic enzyme, the former reacts with some substance in euglobulin in molecular proportions. If this is true, then the quantity of inactive precursor which remains after activation should be inversely proportional to the amount of fibrinolysin added originally. The following experiments were designed to test this possibility.

Fibrinolysin was added in increasing amounts to aliquots of plasma globulin dissolved in buffer. Each mixture was divided into three parts. The first part was assayed for proteo-

lytic activity immediately. The other two parts were allowed to stand in a water bath at 37°C. for 4 hours. Then the enzymatic activity of one of these aliquots was measured; and at this time, 1 cc. of streptococcal fibrinolysin was added to each of the tubes of the third group, and the resultant enzymatic activity assayed immediately.

When proteolytic activity was measured immediately, the activity of the globulin preparation varied directly with the amount of fibrinolysin added until a maximum was reached (Fig. 4). The larger amounts of fibrinolysin seemed to saturate the substance in globulin with which it reacted. After 4 hours, the

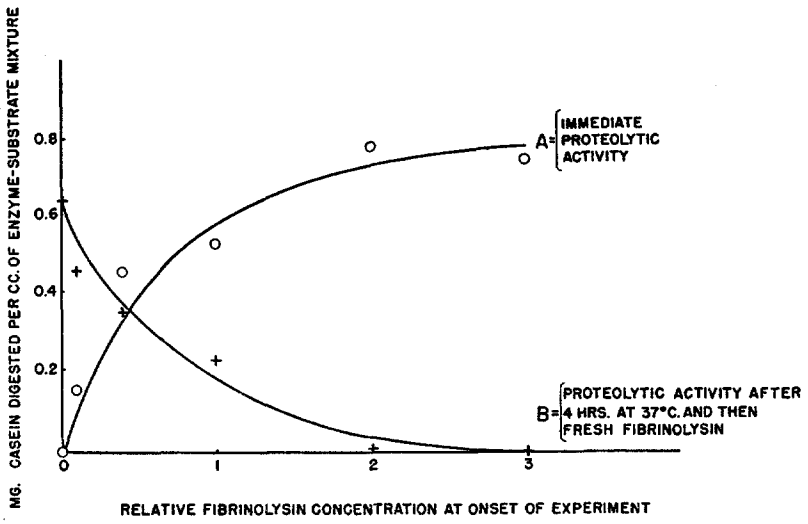

FIG. 4. The effect of additional fibrinolysin on plasma proteolytic enzyme previously activated by fibrinolysin. Varying amounts of fibrinolysin were added to aliquots of globulin solution, and the proteolytic activity measured immediately (curve A). These mixtures were allowed to stand at 37°C. After 4 hours the proteolytic activity of an aliquot of each mixture was again determined. At the same time, 1 cc. of fibrinolysin was added to another aliquot of each mixture and the resultant proteolytic activity was measured. Curve B represents the net proteolytic activity attributable to the addition of fresh fibrinolysin.

enzymatic activity of these preparations decreased considerably, particularly in those tubes to which the smaller amounts of fibrinolysin had been added. When 1 cc. of fibrinolysin was added to each tube after the 4 hour period, there was a great increase in activity in those tubes which originally contained the least fibrinolysin, and no increase in activity in those originally saturated with fibrinolysin. These data, with those described in the preceding section, demonstrate that in the activation of plasma proteolytic enzyme, streptococcal fibrinolysin reacts stoichiometrically with some substance in the globulin preparation.

5. The Effect of Heat or Chloroform on the Activation of Plasma Proteolytic Enzyme by Streptococcal Fibrinolysin.—Christensen and MacLeod (5, 7) re-

ported that the activation of plasma proteolytic enzyme by streptococcal fibrinolysin was catalytic in nature, and in the absence of inhibitory substances the kinetics of activation appeared to be that of a first order unimolecular reaction. A small amount of fibrinolysin was as effective as larger amounts, although the time necessary for complete activation was longer. In the experiments described in the preceding sections, fibrinolysin appeared to react in molecular proportions with some substance in globulin, either enzyme or inhibitor, during the activation of proteolytic enzyme. A small amount of fibrinolysin activated only a small proportion of the enzyme precursor. The following experiments were designed to test whether in the globulin preparation separated by isoelectric precipitation inhibitory substances were present which interfered with the complete activation of all available precursor.

Twenty cc. of pooled serum[1] was heated to 56°C. for 30 minutes, diluted with 19 volumes of water and sufficient 1 per cent acetic acid to bring the pH to 5.2. The resultant precipitate was separated by centrifugation and dissolved in 40 cc. of buffer. Two cc. of globulin solution was placed into each of 4 tubes and varying amounts of streptococcal fibrinolysin (previously dialyzed against cold distilled water and diluted with equal parts of buffer) were added. Sufficient buffer was added to each to bring the volume to 14 cc. The globulin-fibrinolysin mixtures were then incubated at 37°C. The proteolytic activity of the globulin-fibrinolysin mixtures was determined at the onset, and after incubation at 37°C. for 1 and 2½ hours by incubating 2 cc. aliquots with 2 cc. of 0.3 per cent casein at 37°C. for 1 hour. At the same time, the experiment was repeated with the same serum unheated.

When the globulin precipitated from serum which had been heated to 56°C. for half an hour was mixed with small amounts of streptococcal fibrinolysin, the amount of activated enzyme appeared to increase over a period of several hours (Fig. 5, *A*). Furthermore, when sufficient precursor was present, the amount of enzyme activated was directly proportional to the concentration of streptococcal fibrinolysin added. On the other hand, when streptococcal fibrinolysin was added to unheated serum (Fig. 5, *B*) the activity induced by fibrinolysin was maximal at the onset of the experiment and decreased during the period of observation. The same results were obtained by mixing plasma globulin solution with chloroform for 16 hours and removing the chloroform before the addition of streptococcal fibrinolysin.

These experiments imply either that heat or chloroform may activate directly or indirectly plasma proteolytic enzyme or, more likely, that they destroy some substance in the globulin preparation which inhibits the activation of enzyme by streptococcal fibrinolysin. The significance of these observations will be discussed below.

III. The Kinetics of Digestion with Plasma Proteolytic Enzyme

1. The Influence of Substrate Concentration on the Digestion of Casein by Plasma Proteolytic Enzyme.—To determine the influence of varying the con-

[1] Serum was used instead of plasma since heating plasma to 56°C. results in precipitation of fibrinogen.

centration of substrate upon the amount of substrate digested, chloroform-activated enzyme was prepared in the usual manner.

In a typical experiment, 4 cc. of chloroform-activated enzyme, prepared as in other experiments, was added to 4 cc. of casein dissolved in 0.006 N sodium hydroxide. The casein varied in concentration from 0.1 to 0.4 per cent, so that the final concentration of casein in the enzyme-substrate mixture was 0.5 to 2.0 mg. per cc.

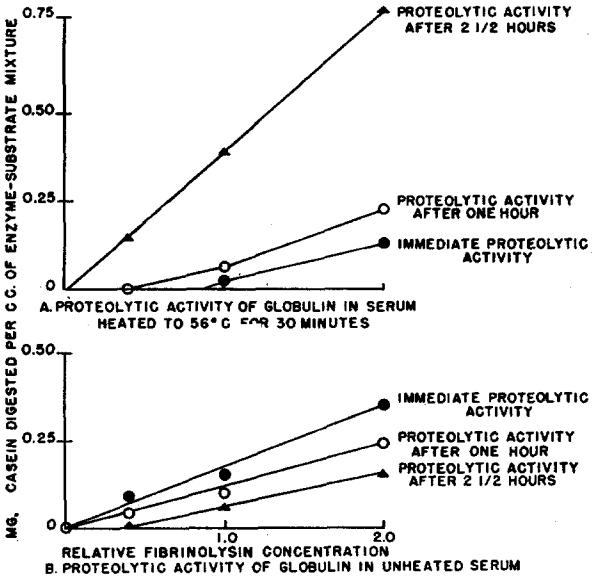

FIG. 5. The proteolytic activity induced by streptococcal fibrinolysin in globulin which had been heated to 56°C. Proteolysis was measured immediately after the addition of fibrinolysin, and again after incubation of the globulin-fibrinolysin mixture at 37°C. for 1, and 2½ hours. See text.

The amount of casein digested during a period of 4 hours is plotted in Fig. 6. During the first hour and a half, the amount of casein digested was independendent of substrate concentration in those tubes with initial casein concentrations of 1.0 mg. per cc. or more. Beyond this time, however, the rate of digestion of casein was directly dependent upon the initial casein concentration.

These studies indicate that the quantitative determination of plasma proteolytic enzyme requires that a concentration of casein be used which would saturate all available enzyme during the period of digestion observed. However, in practice, a concentration of substrate higher than 1.5 mg. per cc. had to be avoided. If the concentration of casein was high relative to the concentration of globulin, when hydrochloric and sulfosalicylic acids were added to assay protein, flocculation and precipitation of protein resulted, instead of uni-

form turbidity. This rendered it impossible to quantify the protein present by the method used. Furthermore, concentrations of casein in the enzyme-substrate mixture higher than 1.5 mg. per cc. apparently inhibited digestion during the first 15 or 20 minutes of incubation. In repeated experiments, when concentrations of casein in the enzyme-substrate mixture of 1.5 mg. per cc. were used, the amount of casein digested was linear until approximately 0.7 mg. per cc. was digested, after which the amount of casein digested per unit time decreased.

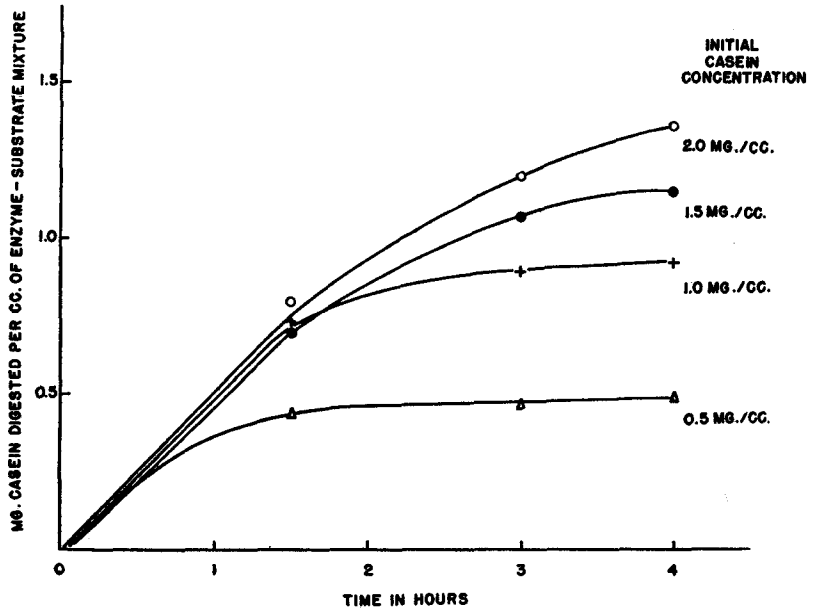

FIG. 6. The influence of the concentration of substrate (casein) on its rate of digestion by chloroform-activated plasma proteolytic enzyme. The initial concentration of casein is expressed in terms of milligrams per cubic centimeter of enzyme-substrate mixture.

2. The Relationship between Concentration of Enzyme and the Digestion of Casein during the 1st Hour of Incubation of Enzyme and Substrate.—When the initial concentration of casein in the enzyme-substrate mixture was 1.5 mg. per cc., digestion with either fibrinolysin- or chloroform-activated enzymes was proportional to the concentration of enzyme present, up to the point where 0.7 mg. per cc. of casein had been digested. Beyond this point, however, the relative rate of casein digested per cc. decreased.

Fig. 7 is a composite of many experiments in which the amount of casein digested by various dilutions of fibrinolysin-activated and chloroform-activated enzyme was plotted against arbitrary units. The amount of enzyme necessary to digest 0.50 mg. of casein per cubic centimeter of enzyme-substrate mixture

in 1 hour was arbitrarily called 50 digestion units. A sample of chloroform-activated enzyme was diluted to varying degrees, and the amount of casein per cubic centimeter of enzyme-substrate mixture digested in 1 hour was determined. The data from one such experiment were then plotted, using the arbitrary point of 50 units = 0.50 mg. as one point on the curve. Using this rough plot, the data from other experiments were fitted to the curve, selecting the best fit to the arbitrary curve. In this manner, it was possible to plot the data

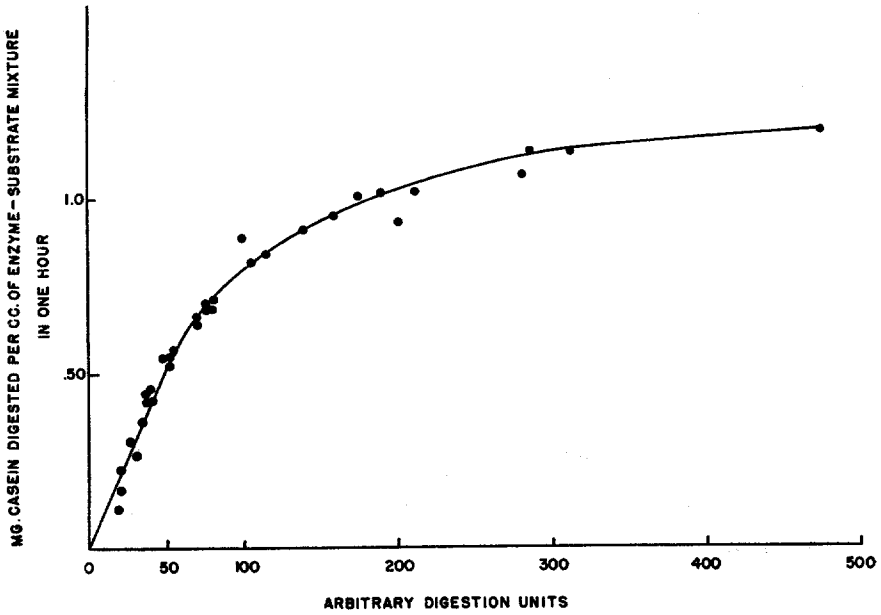

FIG. 7. The influence of the concentration of plasma proteolytic enzyme on the digestion of casein. The data of several experiments have been plotted on a single curve, with 50 digestion units arbitrarily designated as the amount of plasma proteolytic enzyme which will digest 0.50 mg. of casein per cc. of enzyme-substrate mixture in 1 hour, when the initial concentration of casein was 1.5 mg. per cc. of enzyme-substrate mixture. See text.

of many experiments in such a way that arbitrary units would express the proteolytic power of any given specimen. Thus, if a given solution digested 1.00 mg. of casein per cc. of enzyme-substrated mixture in 1 hour, it would contain 195 digestion units of plasma proteolytic enzyme. If the enzyme were diluted with equal parts of buffer, it would contain 97 digestion units and should digest 0.77 mg. of casein per cc. per hour. Actually, these assumptions have been found to be correct in repeated experiments. In this way, by the use of an arbitrary scale, the difficulties imposed by not using a substrate concentration higher than 1.5 mg. per cc. were overcome.

3. The Activity of Plasma Proteolytic Enzyme after the 1st Hour of Incubation

with Substrate.—In studying the activation of plasma proteolytic enzyme by streptococcal fibrinolysin, use was made of the fact that fibrinolysin-activated enzyme prepared from plasma globulin deteriorated rapidly at 37°C. This deterioration appeared to occur early during the 2nd hour after the enzyme had been activated. In these experiments, described in section II 4, the deterioration seemed to take place before the addition of substrate. The following experiments demonstrate that fibrinolysin-activated enzyme also deteriorated in

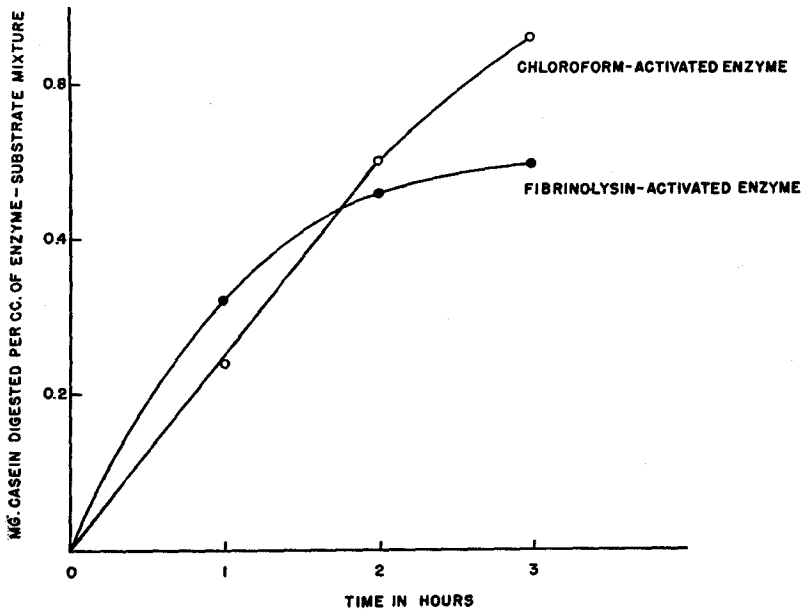

FIG. 8. The relative time of deterioration of chloroform-activated and fibrinolysin-activated plasma proteolytic enzyme. See text.

the presence of casein substrate. On the other hand, chloroform-activated enzyme did not deteriorate appreciably during a period of 3 hours.

The digestion of casein by samples of fibrinolysin-activated and chloroform-activated enzyme has been compared in Fig. 8. Chloroform-activated enzyme did not show appreciable deterioration during the 3 hour period. The rate of digestion decreased only after the amount of substrate fell below the concentration necessary to saturate all available enzyme; that is, when the concentration of casein decreased below approximately 0.8 mg. per cc. This indicates that there was no appreciable deterioration of chloroform-activated enzyme during the period of observation.

On the other hand, in this and many other experiments, the activity of fibrinolysin-activated enzyme decreased rapidly beyond the 1st hour of incu-

bation, regardless of how much casein was still available. This implies that the fibrinolysin-activated enzyme had deteriorated in one way or another. As pointed out before, this decrease in activity occurred even in the absence of substrate. The deterioration of fibrinolysin-activated enzyme occurred in all preparations studied, and became sufficient to affect the assay of proteolytic activity early during the 2nd hour of incubation at 37°C. Ordinarily, proteolytic activity was determined by measuring the amount of substrate digested in 1 hour. Therefore, it was necessary to assay fibrinolysin-activated enzyme immediately after preparation to minimize the effects of deterioration.

4. The Influence of Chloroform on the Deterioration of Plasma Proteolytic Enzyme Activated by Chloroform or by Streptococcal Fibrinolysin.—Chloroform-activated plasma proteolytic enzyme apparently did not deteriorate during several hours' incubation at 37°C. On the other hand, fibrinolysin-activated enzyme deteriorated rapidly. The following experiments were designed to

TABLE III

The Protection of Fibrinolysin-Activated Enzyme by Chloroform

Activator	Time of measurement of activity	Enzymatic activity
		*mg./cc.**
Fibrinolysin	Immediately after preparation	0.76
Fibrinolysin	After incubation at 25°C. for 20 hrs.	0.09
Chloroform	After incubation at 25°C. for 20 hrs.	0.09
Fibrinolysin and chloroform together	After incubation at 25°C. for 20 hrs.	0.60

* Milligrams of casein digested per cubic centimeter of enzyme-substrate mixture in 1 hour.

determine whether chloroform removed or destroyed some substance which hastened the deterioration of plasma proteolytic enzyme.

In a typical experiment, the euglobulin precipitate of 14 cc. of plasma was prepared in two unequal portions. The precipitate of 2 cc. was dissolved in 1 cc. of buffer, and half a cc. of chloroform was added. This mixture was incubated for 20 hours at 25°C., and the chloroform-activated proteolytic activity was then determined in the usual manner. The euglobulin precipitate of the remaining 12 cc. of plasma was dissolved in 6 cc. of broth filtrate containing streptococcal fibrinolysin. It was demonstrated that this amount of fibrinolysin was not sufficient to activate all available precursor. The fibrinolysin-activated proteolytic activity was determined immediately on a 1 cc. aliquot. Another 1 cc. aliquot was allowed to stand at 25°C. for 20 hours, and its proteolytic activity then determined. To a third 1 cc. aliquot was added 0.5 cc. of chloroform and the mixture was allowed to stand at 25°C. for 20 hours, after which its proteolytic activity was determined. It was then possible to compare the proteolytic activity of the globulin immediately after the addition of fibrinolysin, 20 hours after the addition of fibrinolysin, 20 hours after the addition of fibrinolysin and chloroform together, and 20 hours after the addition of chloroform alone.

These data demonstrate that the addition of chloroform to fibrinolysin-activated enzyme immediately after it had been prepared, prevented in large meas-

ure the deterioration of plasma proteolytic enzyme over a period of 20 hours (Table III). It seemed of interest to determine whether chloroform would reactivate fibrinolysin-activated plasma proteolytic enzyme once it had deteriorated.

At the same time as the preceding experiment, two other 1 cc. aliquots of fibrinolysin-activated globulin were incubated at 37°C. for 4 hours. At the end of that time, one aliquot was tested for proteolytic activity. To the other aliquot was added 0.5 cc. of chloroform, the mixture was incubated for 16 hours at 25°C., and its proteolytic activity then determined. It was then possible to compare the proteolytic activity of the globulin immediately after the addition of fibrinolysin; after 4 hours at 37°C. following the addition of fibrinolysin; 16 hours after the addition of chloroform to enzyme which had been incubated for 4 hours at 37°C. after the addition of fibrinolysin; and 20 hours after the addition of chloroform alone.

As can be seen in Table IV, once fibrinolysin-activated enzyme had been allowed to deteriorate, the addition of chloroform did not reverse this deteri-

TABLE IV

Failure of Chloroform to Reverse the Deterioration of Fibrinolysin-Activated Enzyme

Activator	Time of measurement of activity	Enzymatic activity
		*mg./cc.**
Fibrinolysin	Immediately after preparation	0.76
Fibrinolysin	After incubation at 37°C. for 4 hrs.	0.06
Chloroform	After incubation at 25°C. for 20 hrs.	0.09
Fibrinolysin incubated at 37°C. for 4 hrs., then chloroform	After incubation at 25°C. for 16 hrs. following addition of chloroform	0.20

* Milligrams of casein digested per cubic centimeter of enzyme-substrate mixture in 1 hour.

oration. The small increase in activity noted could be accounted for entirely by the activation of precursor not previously activated by fibrinolysin, since the precursor had not been saturated by the amount of fibrinolysin employed.

DISCUSSION

The experiments described define some of the conditions necessary for the optimal activation of plasma proteolytic enzyme by chloroform. Mixtures of chloroform and the euglobulin containing the precursor of plasma proteolytic enzyme were incubated for varying lengths of time and at several temperatures. The greatest proteolytic activity resulted when the plasma euglobulin was incubated with chloroform for 16 to 28 hours at 25°C. This is in contradistinction to the reported results of incubation of chloroform and whole plasma (3, 9), mixtures of which were not maximally active for at least several days. These studies suggest that some substances not in euglobulin may retard the activation of plasma proteolytic enzyme. Incubation of globulin with chloroform at 37°C. resulted in much less proteolytic activity than incubation at 25°C. The explanation of this phenomenon is not clear, although it is possible that

the enzyme may be destroyed by the prolonged incubation at 37°C., perhaps by autodigestion.

The evidence presented did not indicate the mode of activation of plasma proteolytic enzyme by chloroform. Delezenne and Pozerski (3) and later Christensen (9) suggested that chloroform apparently removes some substance which prevents the accumulation of enzyme. Then the enzyme may be activated autocatalytically in the manner in which trypsinogen is activated by trypsin (9). The data support this view but the evidence is not conclusive.

Streptococcal fibrinolysin on the other hand, activated plasma proteolytic enzyme with great rapidity and when maximal amounts of fibrinolysin were used, maximal activity was observed just as early as proteolysis could be measured. Christensen and MacLeod (7) studied the proteolysis of gelatin with a purified streptococcal fibrinolysin prepared from the H46A strain of beta hemolytic streptococcus. They concluded that the activation of plasma proteolytic enzyme by streptococcal fibrinolysin was a catalytic process. The rate of activation was proportional to the concentration of fibrinolysin, but the maximal activity developed was independent of the concentration of fibrinolysin used.

Moreover, Christensen and MacLeod (7, 9) have proposed an hypothesis which explains the data thus far presented concerning the rôle of chloroform and of fibrinolysin in the activation of plasma proteolytic enzyme. Plasma proteolytic enzyme is capable of spontaneous activation. Chloroform removes some substance which destroys the plasma proteolytic enzyme as it is formed. Therefore, when globulin solutions are treated with chloroform, the spontaneously developing enzyme gradually accumulates, and the preparation acquires considerable proteolytic activity. Fibrinolysin, on the other hand, reacts catalytically directly with some substance in globulin to release plasma proteolytic enzyme.

Other data, reported above, however, are not adequately explained by this hypothesis. If the globulin preparation was not previously treated with heat or chloroform, the activation of plasma proteolytic enzyme with streptococcal fibrinolysin behaved as if a stoichiometric reaction occurred. Thus, the proteolytic activity evoked by fibrinolysin acting on plasma globulin preparations not so treated was an hyperbolic function of the concentration of fibrinolysin. This is compatible with the law of mass action. Furthermore, if the concentration of both fibrinolysin and globulin was varied, the proteolytic activity that resulted was proportional to the product of the concentration of fibrinolysin and of globulin. That is, within limits,

$$\frac{[\text{Substance in globulin}] \times [\text{fibrinolysin}]}{[\text{Proteolytic activity}]} = \text{Constant}$$

With the globulin preparation used, the enzymatic activity of fibrinolysin-globulin mixtures began to deteriorate after incubation for about 1 hour at

37°C. This was equally true if small or large amounts of fibrinolysin were used. When preparations of fibrinolysin-activated enzyme were allowed to deteriorate, the addition of fresh fibrinolysin evoked fresh enzymatic activity in those preparations which had low concentrations of fibrinolysin originally, and none in those which had high concentrations of fibrinolysin originally. Therefore, these experiments indicate that fibrinolysin reacted in molecular proportions with some substance in globulin, probably the precursor of plasma proteolytic enzyme itself.

All the data above can be explained by a new hypothesis. The globulin preparation used contained small amounts of inhibitor of activated plasma proteolytic enzyme. Streptococcal fibrinolysin reacts stoichiometrically with the precursor of plasma proteolytic enzyme. In the absence of these inhibitory substances, the enzyme so activated is responsible for further autocatalytic activation of the remaining enzyme precursor. In the presence of inhibitor of the activated enzyme, the autocatalysis is blocked, and the molecular reaction is then apparent.

Certain data described above support this hypothesis. When the plasma globulin was treated with chloroform or heat, streptococcal fibrinolysin now appeared to activate plasma proteolytic enzyme catalytically. However, the globulin thus treated simultaneously lost its ability to inhibit plasma proteolytic enzyme (2). Furthermore, fibrinolysin-activated enzyme prepared from globulin treated with heat or chloroform did not deteriorate with the rapidity of unheated globulin preparations. These experiments suggest that the inhibitory substance which prevents the catalytic activation of plasma proteolytic enzyme may be identical with the inhibitor of activated plasma proteolytic enzyme.

The kinetics of digestion with plasma proteolytic enzyme have not been defined previously. The data presented here demonstrate that the relationship between concentration of enzyme and the amount of substrate digested is similar to that of other hydrolytic enzymes. When the concentration of substrate is sufficiently high to saturate all available enzyme, the rate of digestion by hydrolytic enzymes is directly proportional to the concentration of the enzyme, and to the duration of digestion (10). This is particularly true early in the course of digestion, before the accumulation of inhibitory end products, or a decrease in the amount of substrate available, or the inactivation of the enzyme. The experiments herein described demonstrate that plasma proteolytic enzyme does indeed digest casein in direct proportion to the concentration of enzymes as long as the enzyme is saturated with substrate.

SUMMARY

1. Some conditions for the optimal activation of plasma proteolytic enzyme by chloroform have been described. The activation proceeds slowly. The action of chloroform is probably to remove some substance which inhibits or

inactivates the plasma proteolytic enzyme preparation, rather than a direct activation of the enzyme.

2. Plasma proteolytic enzyme is activated by filtrates of cultures of beta hemolytic streptococci. When streptococcal fibrinolysin is present in maximally effective amounts, the activation is almost instantaneous. When the globulin is prepared from heated serum or the globulin is treated with chloroform, the activation of enzyme by streptococcal fibrinolysin appears to be catalytic. If the globulin is not so treated, the reaction appears to involve a stoichiometric process.

3. The plasma proteolytic enzyme activated by chloroform or by streptococcal fibrinolysin digests casein in direct proportion to the concentration of enzyme and to the time of digestion, during the early period of incubation.

4. Fibrinolysin-activated enzyme deteriorates rapidly relative to chloroform-activated enzyme. This may be due to the removal by chloroform of some substance which inactivates plasma proteolytic enzyme.

It is a pleasure to acknowledge the advice and encouragement of Dr. G. S. Mirick, and Dr. R. M. Archibald.

BIBLIOGRAPHY

1. Dastre, A., *Arch. physiol. norm. et path.*, 1893, **5**, 661.
2. Hedin, S. G., *J. Physiol.*, 1904, **30**, 195.
3. Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, 1903, **55**, 327.
4. Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.
5. Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.
6. Ratnoff, O. D., *J. Exp. Med.*, 1948, **87**, 199.
7. Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.
8. Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Inv.*, 1942, **21**, 533.
9. Christensen, L. R., *J. Gen. Physiol.*, 1946, **30**, 149.
10. Van Slyke, D. D., in Advances in Enzymology and Related Subjects, (F. A. Nord and C. H. Werkman, editors), New York, Interscience Publishers, Inc., 1942, **2**, 33.