STUDIES ON A PROTEOLYTIC ENZYME IN HUMAN PLASMA

I. The Probable Identity of the Enzymes Activated by Chloroform and by Filtrates of Cultures of Beta Hemolytic Streptococci*

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In recent years, interest has been revived in the fibrinolytic properties of human plasma. The rapid lysis of clotted blood has been described in patients with liver disease (1), toxemia of pregnancy (2), shock (3), after typhoid vaccine therapy (4), and pre- and postoperatively (5, 6). The mechanism of fibrinolysis is obscure. Presumably the clot is digested by proteolytic enzymes present in the blood. Any understanding of the problem of clot lysis, therefore, requires an understanding of the proteolytic properties of human plasma. The current study is an investigation of the proteolytic properties of blood, in an attempt to elucidate the mechanism of fibrinolysis.

The proteolytic properties of blood were first noted in 1893 by Dastre (7), who described the digestion of a fibrin clot when it stood in serum for 18 hours. Shortly thereafter, Hedin (8) observed that the blood's proteolytic properties were contained in the euglobulin fraction of plasma which was precipitated with dilute acetic acid. At the same time, Delezenne and Pozerski (9) observed that the addition of chloroform to serum activated a proteolytic enzyme. This chloroform-activated proteolytic property of blood was shown by Tagnon (10) to reside in the acid-insoluble euglobulin fraction, as in the case of the enzymatic activity which was present spontaneously.

Tillett and Garner (11), in 1933 observed that bacteria-free filtrates of cultures of beta hemolytic streptococci contained a water-soluble substance which could not be dialyzed through a semipermeable membrane and which caused rapid lysis of plasma or fibrin clots. This remarkable property was shown by Milstone (12) to depend upon the presence of some substance which he called "lytic factor" in the acid-insoluble euglobulin of plasma. Kaplan (13) and Christensen and MacLeod (14, 15) demonstrated that the same fraction of plasma contained lytic factor and the chloroform-activated proteolytic enzyme. Their studies offered strong evidence that the fibrinolytic properties of streptococcal filtrates were due to the activation of a plasma proteolytic enzyme,

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and that the enzyme so activated was identical with that activated by chloroform.

The present study introduces the use of new quantitative methods for the determination of plasma proteolytic activity, employing the nephelometric estimation of the protein concentration of the enzyme-substrate mixture, as suggested by Grob (16). Using this new method, it has been possible to confirm the probable identity of the plasma proteolytic enzyme activated by chloroform and by filtrates of beta hemolytic streptococci. In the accompanying paper (17) new data will be presented which seem to indicate hitherto undescribed kinetic mechanisms in the activation of plasma proteolytic enzyme.

Methods

Nomenclature (*15, 18, 19*).—The proteolytic enzyme of plasma or serum has been called plasma (or serum) protease, tryptase, trypsin, fibrinolysin, plasmin, or proteinase. In an effort to prevent confusion, the term plasma proteolytic enzyme will be used throughout. The precursor of this enzyme has been termed plasminogen, tryptogen, or profibrinolysin, but here the term plasma proteolytic enzyme precursor will be used. The enzyme when activated by chloroform will be distinguished as chloroform-activated enzyme. The fibrinolytic principle of cultures of beta hemolytic streptococci was originally called fibrinolysin, and more recently streptokinase. One could speak of the active principle as streptococcal plasma proteolytic enzyme activator. Since this term is cumbersome, we shall use the original name, fibrinolysin, and the enzyme activated by filtrates of cultures of hemolytic streptococci will be termed fibrinolysin-activated enzyme. In this and the paper to follow the term enzyme will be used to mean the crude enzyme preparation used, but does not imply a purified product or even a single substance.

Venous blood was collected under sterile conditions from the antecubital vein of human subjects and transferred immediately to a 50 cc. centrifuge tube containing 0.2 cc. of an oxalate solution for each 5 cc. of blood drawn. The oxalate solution was a mixture of 1.2 gm. of ammonium oxalate and 0.8 gm. of potassium oxalate dissolved in 100 cc. of water (20). The plasma was immediately separated by centrifugation.

Preparation of Enzyme.—The spontaneous proteolytic activity of plasma was observed by Hedin to be due to the presence of some substance in the euglobulin fraction precipitated by dilute acetic acid. Tagnon (10) reported that the same fraction contained the proteolytic enzyme activated by chloroform; and Milstone (12) demonstrated that a similar fraction was necessary for the fibrinolytic action of streptococcal filtrates.

In most of the experiments described in this report, a euglobulin fraction was separated in the following manner. Plasma was added to distilled water in the ratio of 1 cc. of plasma to 19 cc. of water, and the solution was acidified with 1 per cent acetic acid to the pH desired, usually 5.2, as measured with a Beckman laboratory model G pH meter. The resultant precipitate was separated by centrifugation and dissolved in 0.85 per cent sodium chloride solution buffered by M/20 potassium phosphate at pH 7.35 (buffer) usually in one-fourth the original plasma volume. This globulin solution was then activated either by chloroform or by hemolytic streptococcal filtrates.

(*a*) *Chloroform-Activated-Enzyme:* The optimal preparation of chloroform-activated enzyme required meticulous attention to a number of factors. In most experiments, chloroform was added to the globulin solution, and the tube stoppered with a rubber stopper and placed in a water bath at 25°C. for 17 hours. Immediately upon addition of chloroform a heavy white precipitate formed in the globulin solution, usually followed sometime within

an hour by a gray gelatinous clot. This dissolved in about 8 hours, leaving a white granular precipitate. After 17 hours the contents of the tube were transferred to a graduated centrifuge tube and diluted with the buffered saline solution to a suitable volume, usually 10.0 cc. The mixture was then centrifuged for 10 minutes at 2000 R.P.M. in an angle centrifuge (rim diameter = 10 inches). The grayish supernatant solution contained chloroform-activated enzyme.

(*b*) *Fibrinolysin-Activated Enzyme:* Fibrinolysin, the plasma proteolytic enzyme activator in streptococcal filtrates, was prepared from the CO strain of group A, or the H46A strain of group C beta hemolytic streptococcus.[1] Trypticase soy media, Baltimore Biological Laboratories (a tryptic digest of casein, 17 gm., papaic digest of fat-free soy meal, 3 gm., sodium chloride, 5 gm., dipotassium phosphate, 2.5 gm., and dextrose, 2 gm. per liter of media), were inoculated with 1 cc. of an 8 hour broth culture of streptococci for each 100 cc. of media. After 14 hours, 1 N sodium hydroxide was added to the culture at intervals to keep the pH between 7.0 and 7.8 (14). After 24 hours the culture, now profuse, was centrifuged and the supernatant fluid filtered through a Seitz filter. The filtrate, containing streptococcal fibrinolysin in crude form, was used as a source of this material in the experiments described below. However, all experiments were confirmed with a more purified preparation made by dialyzing the broth filtrate in a cellophane casing against frequent changes of distilled water at 4°C. over a period of 3 days, and then concentrating the dialysate by evaporation in the casing to a volume one-fifth of the original.

Fibronolysin-activated enzyme was prepared by the addition of suitable amounts of fibrinolysin-containing filtrates of streptococcal cultures to the buffered globulin solution.

Preparation of Substrate.—In most experiments, the substrate used was an aqueous solution of casein (vitamin-test, Smaco), in a concentration of 0.3 per cent. The casein was dissolved in sufficient 1 N sodium hydroxide to produce a final concentration of 0.004 or 0.006 N, by frequent stirring and gentle heating, never above 56°C. A few drops of toluol were added as a bacteriostatic agent, and the casein solution stored at 4°C.

Measurement of Proteolysis.—Proteolytic activity was measured by adding the solution of substrate to an equal volume of activated enzyme and incubating the mixture in a water bath at 37°C. This temperature was shown by Christensen and MacLeod (15) to be optimal for digestion of casein and gelatin by plasma proteolytic enzyme. In experiments lasting over 3 hours, a few drops of toluol were added as a bacteriostatic agent. The progress of digestion was observed by removing 1 cc. aliquots at suitable intervals and measuring the precipitable protein nephelometrically. This was done with a Coleman, Jr., spectrophotometer at a wave length of 420 mμ, using cuvettes with an internal diameter of 10 mm. To each 1 cc. aliquot, 2 cc. of 12.5 per cent hydrochloric acid was added rapidly, followed by 0.5 cc. of 20 per cent sulfosalicylic acid. To insure uniform turbidity, it was necessary to add the acids as rapidly as possible. Maximal turbidity developed in about 5 minutes. At 420 mμ, the degree of turbidity obeys the Lambert-Beer law to an optical density of approximately 0.650, and concentrations of enzyme and substrate which exceeded this density were avoided. Unless globulin was present, the turbidity decreased rapidly upon addition of sulfosalicylic acid due to the formation of large aggregates. This made it difficult to estimate the concentration of protein quantitatively in the absence of globulin. For the same reason, high concentrations of casein could not be used. The amount of substrate digested was calculated by subtracting the final from the initial optical density of the precipitated protein solutions, and converting the difference in optical density to milligrams of casein. With the instrument and cuvettes used, a solution of casein containing 0.100 mg. per cc. had an

[1] These strains were furnished through the courtesy of Dr. Eleanor Bliss and Dr. L. M. Christensen respectively.

optical density of 0.225. The conversion of differences in optical density to milligrams of casein assumes that none of the globulin is digested during the course of the experiment. Actually in repeated experiments, this was substantially true. At most, the digestion of the activated globulin mixture used accounted for an equivalent of 0.03 to 0.04 mg. of casein.

EXPERIMENTAL

1. The Point of Maximal Precipitation of Chloroform-Activated and Fibrinolysin-Activated Plasma Proteolytic Enzyme.—The point of maximal precipitation of the euglobulin containing the precursor of plasma proteolytic enzyme has not been clearly defined. Tagnon (10) reported that the chloroform-activated proteolytic activity of plasma was present in the euglobulin fraction precipitated by dilute acetic acid at pH 6.0. The euglobulin which Milstone (12) found necessary for the action of fibrinolysin was precipitated by acidifying serum diluted to 20 times its volume in distilled water with 0.32 volumes of acetic acid. This procedure, however, may result in a different pH with each serum. Thus, when 0.5 cc. of 1 per cent acetic acid was added to 2 cc. of plasma diluted with 38 cc. of water, the pH of the resultant mixture varied, in eight experiments, from 5.0 to 5.7. Kaplan (13) and Christensen and MacLeod (14, 15) demonstrated the probable identity of the fractions containing the enzymes activated by chloroform and by fibrinolysin. The following data show that the globulin containing the precursors of chloroform-activated and fibrinolysin-activated enzymes is precipitable from dilute solution maximally at pH 5.2 rather than at pH 6.0.

In a typical experiment, 2 cc. of plasma was added to each of 8 tubes containing 38 cc. of distilled water. Varying quantities of 1 per cent acetic acid were added to each tube so that the pH of the mixture ranged from 6.20 to 4.80. Each tube was then treated in the following manner: The resultant precipitate was separated by centrifugation, the supernatant fluid discarded, and the precipitate dissolved in 1.0 cc. of buffer. Half a cc. of chloroform was then added to each tube, and the mixture incubated at 25°C. for 17 hours. The contents of each tube were then diluted with buffer to a volume of 5 cc. An equal volume of 0.3 per cent casein solution in 0.004 N sodium hydroxide was added, and the mixture incubated at 37°C. for 2 hours. Similarly the globulins precipitated at hydrogen ion concentrations between pH 6.3 and 4.8 were dissolved in 1 cc. of fibrinolysin-containing streptococcal filtrate, and then diluted to 15 cc. with buffer. Five cc. of 0.3 per cent casein was added to a 5 cc. aliquot and the amount of casein digested during 1 hour at 37°C. determined.

As can be seen in Fig. 1, maximal proteolytic activity was present in the globulin precipitated at pH 5.2, whether the enzyme was activated by the fibrinolysin in streptococcal filtrates, or by chloroform. That is, the precursor of the enzyme which was activated by chloroform could not be distinguished from that which was activated by streptococcal fibrinolysin by the use of this technique. Repeated experiments showed that trypticase soy media itself and the streptococcal fibrinolysin in the absence of plasma globulin had no proteolytic activity. Furthermore, the casein solution alone did not hydrolyze measurably when incubated for 24 hours at 37°C.

As one might predict from the variation in the buffering capacity of plasma from person to person, the volume of acetic acid which must be added to diluted plasma to bring the solution to pH 5.2 must be determined separately for each sample.

2. The Optimal pH for the Digestion of Casein by Chloroform-Activated and Fibrinolysin-Activated Plasma Proteolytic Enzyme.—The proteolytic enzyme of plasma is most efficient at the hydrogen ion concentrations likely to be found in blood. Schmitz (21) reported that spontaneously activated plasma proteolytic enzyme digested fibrin most completely when the hydrogen ion concentration of the mixture ranged between pH 7.0 and 8.0. Christensen and MacLeod (15) observed that the proteolytic enzyme of blood activated either by chloroform or by streptococcal fibrinolysin digested gelatin optimally at hydrogen ion concentrations between pH 7.2 and 7.6. The following experiments in which casein was used as a substrate, corroborate these reports.

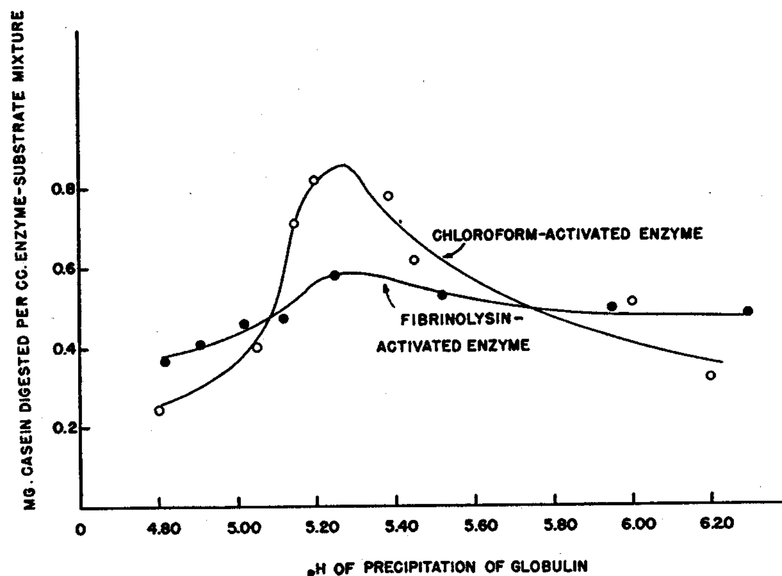

FIG. 1. The point of maximal precipitation of the plasma euglobulin fraction containing proteolytic enzyme precursor as determined by the pH of maximal precipitation. See text.

In a typical experiment, the euglobulin precipitated at pH 5.2 from 2 cc. of pooled plasma was dissolved in 1 cc. of buffer. Half a cc. of chloroform was added and the mixture was incubated for 17 hours at 25°C. The contents of two such tubes were then combined and diluted with buffer to 12 cc. The chloroform and precipitate were then removed by centrifugation. One cc. of the supernatant fluid was placed in each of 11 tubes which contained 4 cc. of M/20 potassium phosphate buffered to various hydrogen ion concentrations between pH 3.3 and 9.7. To each tube was then added an equal volume of 0.3 per cent casein solution in 0.006 N sodium hydroxide. The mixture was then incubated at 37°C. The pH of the

enzyme-substrate mixture was determined with a Beckman pH meter approximately 15 minutes after the mixture was made.

Fibrinolysin-activated enzyme was prepared by dissolving the euglobulin precipitate from 4 cc. of plasma in 2 cc. of streptococcal fibrinolysin, and diluting the solution to 12 cc. with 0.85 per cent sodium chloride buffered at pH 7.35 with potassium phosphate. One cc. was then placed in each of 11 tubes containing 4 cc. of the potassium phosphate buffers. An equal volume of casein was added, the mixture incubated at 37°C., and the pH of the enzyme-substrate mixture measured after 15 minutes.

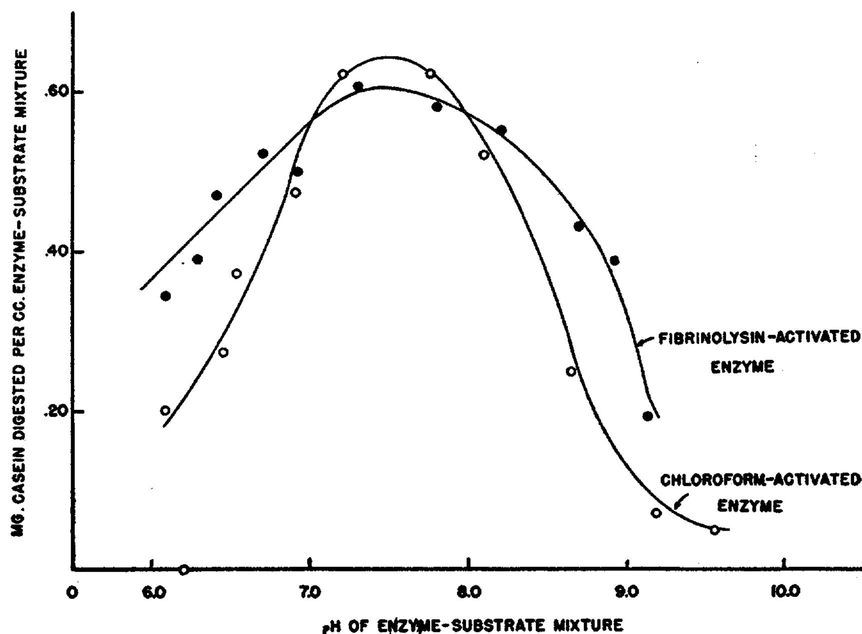

FIG. 2. The optimal pH of the enzyme-substrate mixture for the digestion of casein by plasma proteolytic enzyme, activated by fibrinolysin and by chloroform. See text.

These experiments demonstrated that a crude preparation of plasma proteolytic enzyme, whether activated by chloroform or by streptococcal fibrinolysin digested casein maximally at hydrogen ion concentrations between pH 7.0 and 8.0 (Fig. 2). In all subsequent experiments, therefore, the pH of the enzyme-substrate mixture was kept within these limits. The hydrogen ion concentration of a mixture of an equal volume of casein in 0.004 N or 0.006 N sodium hydroxide and globulin dissolved in pH 7.35 buffer was uniformly between pH 7.5 and 8.0.

3. The Action of Fibrinolysin-Activated and Chloroform-Activated Plasma Proteolytic Enzyme against Various Substrates.—The spontaneously active proteolytic enzyme in plasma was first observed as a result of its ability to digest a fibrin clot (9). This enzyme has also been said to digest casein and gelatin

but not egg albumin (8). When plasma proteolytic enzyme is activated by chloroform, the same substrates are said to be digested (9) and in addition, fibrinogen (22) and hemoglobin (13). Kaplan (13) and Christensen (14) pointed out that the proteolytic enzyme of plasma activated by streptococcal fibrinolysin digested the same substrates. As shown in Table I, the enzyme activated by either method attacked casein and crystalline zinc insulin. With the nephelometric methods used, bovine albumin (fraction V of Cohn), pumpkin seed globulin, egg albumin, lactalbumin, and edestin were not digested by the enzyme activated by either method.

TABLE I

The Digestion of Various Substrates by Plasma Proteolytic Enzyme

Substrate	Concentration of substrate in enzyme-substrate mixture	Amount of substrate digested per cc. of enzyme-substrate mixture in 1 hr.	
		Fibrinolysin-activated enzyme	Chloroform-activated enzyme
Casein	1.5 mg./cc.	0.85 mg.	0.26 mg.
Zinc insulin crystals (Lilly)	40 units/cc.	1.6 units	0.7 units
Bovine albumin (fraction V)	1.25 mg./cc.	0	0
Pumpkin seed globulin	1.0 mg./cc.	0	0
Egg albumin	2.5 mg./cc.	0	0
Lactalbumin	0.25 mg./cc.	0	0
Edestin	0.25 mg./cc.	0	0

No significant difference, then, was noted between the behavior of plasma proteolytic enzyme preparations, activated by chloroform and those activated by streptococcal fibrinolysin, towards various substrates. Repeated experiments, however, showed that the enzyme activated by fibrinolysin was uniformly more active than that activated by chloroform.

4. The Effect of Adding Streptococcal Fibrinolysin to Chloroform-Activated Plasma Proteolytic Enzyme.—The hypothesis that the chloroform-activated and fibrinolysin-activated plasma proteolytic enzymes are identical assumes that the precursors of the two enzymes are identical. Therefore, total activation by either method should remove all the available precursor. Experiments of Christensen (23) showed that this was probably true. The following experiments were designed further to test this hypothesis.

In a typical experiment, globulin was prepared from 4 cc. of plasma. The fibrinolysin-activated proteolytic activity was determined on exactly one-third of this globulin solution by adding 0.1 or 0.2 cc. of streptococcal fibrinolysin, amounts much less than necessary to activate all the precursor present. The remaining globulin was treated with chloroform for 17 hours in the usual manner. At the end of that time, the enzymatic activity was determined on exactly half the specimen. To the other half, fibrinolysin was added in the same

TABLE II

Digestion of Casein by Fibrinolysin-Activated Enzyme, Chloroform-Activated Enzyme, and Enzyme Activated by Chloroform and Fibrinolysin in Succession

Preparation	Enzyme activated by fibrinolysin (A)	Enzyme activated by chloroform (B)	Calculated sum of A and B	Enzyme activated by chloroform followed by fibrinolysin
	*mg./cc.**	*mg./cc.**	*mg./cc.**	*mg./cc.**
11-21-46	0.34	0.37	0.71	0.81
11-25-46	0.74	0.23	0.97	0.95
1- 4-47	0.59	0.26	0.85	0.89
1- 8-47	0.74	0.09	0.83	0.89
2- 7-47	0.44	0.17	0.61	0.75
2-11-47	0.37	0.30	0.67	0.70
2-13-47	0.11	0.20	0.33	0.43

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

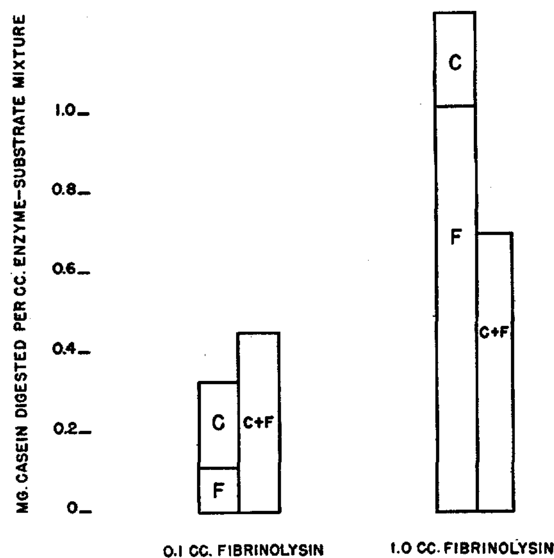

FIG. 3. The activation of plasma proteolytic enzyme by chloroform, by streptococcal fibrinolysin, and by combinations of the two. This graph illustrates the proteolytic activity resulting from treatment of a globulin preparation with chloroform (*C*) and with fibrinolysin (*F*). This is compared with the enzymatic activity of the globulin preparation treated successively with the same amounts of chloroform and fibrinolysin (*C* + *F*). With small amounts of fibrinolysin, the enzymatic activity evoked by the combination was roughly the sum of the activities of the globulin treated with chloroform or fibrinolysin alone. With large amounts of fibrinolysin the activity evoked by chloroform followed by fibrinolysin was less than that evoked by fibrinolysin alone.

concentrations as before, and the resultant activity measured. The concentration of globulin was the same in all preparations in the final enzyme-substrate mixture.

These experiments demonstrated that the enzymatic activities induced by chloroform and by small amounts of fibrinolysin were approximately additive (Table II). In most preparations the activity resulting from successive treatment with the two agents was actually a little greater than the sum of the separate enzymatic activities. This may be because treatment of globulin with chloroform retards the deterioration of fibrinolysin-activated enzyme, promotes increased activation of enzyme by streptococcal fibrinolysin, or both. These possibilities are discussed in the accompanying paper (17).

Christensen (14) observed that the addition of concentrated fibrinolysin to chloroform-activated plasma proteolytic enzyme resulted in proteolytic activity less than that produced by fibrinolysin alone. Similarly in the experiments reported here, when fibrinolysin alone was used in quantities sufficient to activate all available plasma proteolytic enzyme, more casein was digested than was the case when the same amount of fibrinolysin was added to the same amount of globulin, previously activated by chloroform. That is, the enzymatic activities evoked by chloroform and fibrinolysin were no longer additive.

The results of such an experiment are represented graphically in Fig. 3. The proteolytic activity induced by chloroform followed by a small amount of fibrinolysin was somewhat greater than the sum of the activities of a preparation activated by chloroform and another activated by the same amount of fibrinolysin. The enzymatic activity induced by chloroform followed by a large amount of fibrinolysin, on the other hand, was actually less than that produced by the same amount of fibrinolysin alone. A possible explanation of this finding will be discussed below.

DISCUSSION

The data presented support the view that the plasma proteolytic enzymes activated by streptococcal fibrinolysin and by chloroform are identical. This conclusion is suggested by experiments which show that the precursor of the enzyme activated in either manner is present in the same acid-insoluble euglobulin; that the enzyme activated in either manner digests casein optimally at hydrogen ion concentrations between pH 7.0 and 8.0; that the same substrates are digested; and that the enzyme can be activated successively by both agents, but only until the available precursor is exhausted.

A crude separation of the enzyme was made by acid precipitation. The precursor of plasma proteolytic enzyme was present in the euglobulin fraction precipitated at pH 5.2. This fraction is, of course, a mixture of many substances, and includes such proteins as fibrinogen and prothrombin, as well as the precursor of plasma proteolytic enzyme.

The optimal hydrogen ion concentration for the digestion of casein by plasma proteolytic enzyme is between pH 7.0 and 8.0. This hydrogen ion concentra-

tion approximates that previously described by Schmitz (21) and Christensen and MacLeod (15) for the digestion of fibrin and gelatin. Trypsin similarly acts optimally at an approximately neutral pH. Earlier workers described the plasma proteolytic enzyme as "plasma trypsin," but the experiments of Kaplan (24) and Christensen and MacLeod (15) clearly demonstrate that the plasma proteolytic enzyme and trypsin are not identical.

The substrates digested by plasma proteolytic enzyme include fibrinogen, fibrin, gelatin, hemoglobin, casein, and crystalline zinc insulin. Prothrombin too is apparently destroyed by plasma proteolytic enzyme, though the evidence for this is inferential (22). No difference was observed in the substrates attacked by the enzyme activated in either manner.

When small amounts of streptococcal fibrinolysin were added to plasma proteolytic enzyme already activated by chloroform, the preparation increased in activity. The increase in activity was approximately equal to the proteolytic activity induced by fibrinolysin acting alone on the same euglobulin preparation. That is, the activities induced by chloroform and by small amounts of fibrinolysin were additive. When fibrinolysin was used in concentrations sufficient to activate plasma proteolytic enzyme maximally, the proteolytic activities of chloroform-activated and fibrinolysin-activated enzymes were no longer additive. In fact, the addition of such an amount of fibrinolysin to chloroform-activated enzyme actually resulted in less activity than that produced by the same amount of fibrinolysin alone. These experiments imply that the precursors of fibrinolysin-activated and chloroform-activated enzymes are identical or, less likely, that some substance in plasma is necessary for the activation of each. The precursor may be activated by chloroform, but during the period of observation all the precursor cannot be so activated. The remaining precursor may be activated by streptococcal fibrinolysin. Therefore, maximal amounts of fibrinolysin added to globulin solution previously activated by chloroform cannot activate more precursor than can be activated by the same amount of fibrinolysin alone.

In the experiments described, large amounts of fibrinolysin added to chloroform-activated enzyme actually evoked less enzymatic activity than that evoked by the same amount of fibrinolysin alone. This may be because chloroform may not only activate precursor, but also denature some of the precursor or enzyme as well (23). Another mechanism, however, is possible. Perhaps some of the chloroform-activated enzyme had already deteriorated by the time fibrinolysin was added. Once deteriorated, the enzyme may be incapable of reactivation by fibrinolysin. Experiments with fibrinolysin-activated enzyme lend force to this suggestion. In the accompanying paper, it will be shown that fresh fibrinolysin did not reactivate fibrinolysin-activated enzyme once this had deteriorated (17).

SUMMARY

1. The experiments reported suggest that the plasma proteolytic enzyme activated by streptococcal fibrinolysin is identical with that activated by chloroform.

2. The precursor of this plasma proteolytic enzyme is precipitated with the euglobulin fraction of plasma at pH 5.2.

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