A PROTEOLYTIC ENZYME OF SERUM: CHARACTERIZATION, ACTIVATION, AND REACTION WITH INHIBITORS*

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(Received for publication, April 6, 1945)

INTRODUCTION

In previous reports (Christensen, 1944, 1945) evidence has been presented which indicates that the lysin factor of Milstone (1941) is an inactive proteolytic enzyme of serum which may be activated by streptococcal fibrinolysin, and that the activation is apparently catalytic in nature. It has also been shown that proteolysis by this activated enzyme is responsible for the phenomenon of streptococcal fibrinolysis.

Dastre (1893) originally drew attention to the proteolytic activity of serum, and a few years later Delezene and Pozerski (1903) reported that serum could be rendered proteolytic by treatment with chloroform. Since that time, many reports have appeared dealing with this chloroform-activated serum protease. In a series of recent papers, Tagnon and his coworkers (1942 *b*, *c*) have reviewed much of the literature. The serum protease may also be activated by other means, such as treatment with organic solvents or dialysis (Yamakawa, 1918), acid precipitation of the serum (Opie and Barker, 1908), and by treatment with cerain organic compounds such as urea, benzoate, thiocyanate (Jühling and Wöhlisch, 1938), and cresols (Pope, 1938). The rôle of the serum protease in blood coagulation has been investigated by Ferguson (1939, 1940) and by Tagnon (1942 *a*, *b*, *c*, *d*).

Delezene and Pozerski (1903) observed that serum contains two opposing elements, the chloroform-activated protease, and an inhibitor of proteases, first described by Hildebrandt (cited by Opie, 1922). They were led to the conclusion that serum protease is normally inactive because of the action of the serum inhibitor, and that the function of the chloroform is to free the protease from it. The concept that serum protease becomes active because of the removal or inactivation of inhibitor has been held by several investigators. Yamakawa (1918) for example, suggested that the activation of serum protease upon treatment with organic solvents is due to removal of the inhibitor, a conclusion supported by the work of Teale and Bach (1919) who showed that inhibitor is removed from serum as denaturation of the serum proteins takes place. Schmitz (1937) interpreted the increase in activity which he noted when serum extracts were treated with alumina gel or dialyzed in dilute acetic acid to be due to the splitting of the inhibitor-enzyme complex, the inhibitor being adsorbable,

* This work was supported in part by the Commission on Pneumonia, Board for the Investigation and Control of Influenza and other Epidemic Diseases in the United States Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

acid-soluble, and dialyzable. Jühling and Wöhlisch (1938) concluded that the fibrinolysis which follows treatment of plasma with urea is due to removal of the inhibitor by urea. The literature on the serum inhibitor of proteases has been reviewed by Opie (1922) and by Grob (1943).

Schmitz (1938), employing the method used by Kunitz and Northrop (1936), isolated a small amount of inhibitor from serum which appeared to be similar to, if not identical with the crystalline trypsin inhibitor isolated by the latter workers from the pancreas.

There are certain similarities between fibrinolysin-activated lysin factor and chloroform-activated serum protease (Christensen, 1944, 1945; Kaplan, 1944). For example both are present in the euglobulin fraction of serum, both are present as inactive precursors which must be activated, and both are active against a variety of proteins at neutral pH.

Despite the similarities between chloroform-activated serum protease and fibrinolysin activated lysin factor, one striking difference may be noted. Activation of the serum protease by any of the recommended methods appears to be a relatively slow process, whereas in the presence of an excess of fibrinolysin, lysin factor is activated almost instantaneously. These observations have been confirmed recently by Kaplan (1944).

The data obtained in the present study indicate that the protease activated by chloroform and that activated by fibrinolysin are one and the same enzyme. It is also shown that the protease is not inactive because of combination with inhibitor, but because it is in an inactive, precursor state from which it can be activated by streptococcal fibrinolysin in a manner analogous to the activation of trypsinogen by enterokinase or the mold kinase of Kunitz (1938). Following activation, the active enzyme may be inhibited by serum inhibitor or crystalline trypsin inhibitor obtained from the pancreas. This inactivation, however, is not influenced by fibrinolysin. The characteristics of the serum enzyme as determined in the present study indicate that the protease is not identical with pancreatic trypsin.

Methods and Materials

1. The preparation and partial purification of streptococcal fibrinolysin concentrates have been described in detail in a previous paper (Christensen, 1945). The concentrated fibrinolysin solutions used in the present study represent a 200- to 300-fold concentration of a culture supernatant.

2. The method of determining proteolytic activity has been described in detail in a previous paper (Christensen, 1945). The proteolytic unit used in the present paper is defined as the amount of protease which will cause a decrease of 1 per cent per minute in the specific viscosity of the reaction mixture of gelatin and enzyme. The rate of viscosity drop is linear for about the first 10 to 15 minutes and is directly proportional to the enzyme concentration over the ranges used in this study.

3. Lysin Factor.—Lysin factor preparations containing only small amounts of serum

inhibitor were prepared by dialyzing human serum[1] for 1 to 2 days against running tap water, followed by dilution with 2 volumes of distilled water. Upon acidification to pH 5.0–5.5 with acetic acid a flocculent precipitate quickly settled out. The precipitate was collected by centrifugation and dissolved in a volume of saline buffer at pH 7.4 equal to ^{1} the original serum volume.

4. Activated Lysin Factor.—One volume of concentrated lysin factor solution was mixed with 1 volume of 1:10 dilution of concentrated fibrinolysin and incubated at 37°C. for 10 minutes. Under these conditions all of the proteolytic activity of the lysin factor is liberated within the first few minutes. The activated lysin factor solutions are stable for several hours at room temperature and for much longer periods in the refrigerator. However, in the majority of the experiments reported in the present paper, lysin factor was activated immediately before use.

5. Serum Protease.—The method employed was essentially that described by Tagnon (1942 *b*), a modification of the process devised by Delezene and Pozerski (1903). With lots of plasma of 50 to 100 ml., fairly consistent results were obtained. When the same method was used with 1 to 5 liter quantities of plasma, however, activation was not obtained consistently, and not more than 25 to 50 per cent of the preparations showed sufficient activity to be useable. The details of the preparative procedure were as follows: Plasma[1] was shaken with $\frac{1}{10}$ volume of chloroform for 2 minutes. A fibrin clot formed within a few hours and dissolved again within 24 to 36 hours, although in some cases a longer time was required. The majority of active preparations were obtained by fractionation of the material 2 to 4 days after disappearance of the fibrin clot, although in one instance activity did not develop for more than a week. The chloroform-serum mixture was centrifuged for 1 to 2 hours and the supernatant decanted from the mixture of chloroform and denatured proteins. The supernatant was then treated exactly as in the method for the preparation of lysin factor, namely, acidification to pH 5.0–5.5 of the dialyzed and diluted material. The precipitate was dissolved in saline buffer in a volume equivalent to $\frac{1}{10}$ the original plasma volume at pH 7.4.

It is evident from the foregoing that lysin factor and serum protease are present in the same fraction of serum, the euglobulin.

6. Crystalline Trypsin[2].—The sample of crystalline trypsin contained about 50 per cent magnesium sulfate. A solution containing 1.0 mg. per ml. of the dry material was made up in 0.05N HCl. Solutions of the concentration desired for testing were made up immediately before use in saline buffer and adjusted to pH 7.4.

7. Crystalline Trypsin Inhibitor[3].—The crystalline inhibitor preparation contained about 50 per cent magnesium sulfate. The dry material was dissolved in saline buffer,

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[1] The majority of the human plasma used in the present study was made available by the Blood Donor Service of the New York Chapter of the American Red Cross. It represented for the most part samples of whole blood which could not be used by the Donor Service because of positive serology, a short collection, or because it was of a type not suitable for whole blood transfusion.

[2] The crystalline trypsin was supplied through the kindness of Dr. J. S. Fruton.

[3] The crysalline trypsin inhibitor was supplied through the kindness of Dr. J. H. Northrop and Dr. M. Kunitz.

pH 7.4, in a concentration of 1.0 mg. per ml. This stock solution is stable in the refrigerator for a week or more. Before use, dilutions were made to the required concentration in saline buffer.

8. Serum Inhibitor Solution.—Landsteiner (1900) and others (Hedin, 1904–05; Opie and Barker, 1907; Hussey and Northrop, 1923) have shown that the inhibitory activity of serum against proteases is associated with the albumin fraction. The serum inhibitor used in these experiments consisted of a twofold concentration of the albumin fraction of pooled human sera, partially purified by several reprecipitations followed by dialysis. The final solution was made up in saline buffer, pH 7.4.

9. Quantitative Estimation of Serum Inhibitor.—Grob (1943) has employed a method for the estimation of serum inhibitor based on its ability to inhibit the tryptic digestion of casein. In the present study serum inhibitor was measured by comparing the inhibitory action of the serum sample and a standard preparation of crystalline trypsin inhibitor on the tryptic digestion of gelatin. A standard inhibition curve was prepared by mixing 0.5 ml. of solutions containing varying amounts of a standard crystalline trypsin inhibitor solution with 0.5 ml. of a solution of a standard crystalline trypsin solution containing 0.01 mg. per ml. The mixtures were incubated for 15 minutes and the residual proteolytic activity determined by the method used for the determination of serum protease activity. When the logarithm of the residual proteolytic activity is plotted against the concentration of trypsin inhibitor, a straight line is obtained. In determining the inhibitory activity of an unknown solution, 0.5 ml. quantities of appropriate dilutions of the sample were mixed with 0.5 ml. of the standard crystalline trypsin solution and incubated for 15 minutes. The residual activity of the mixture is then determined, and by interpolation on the standard curve the inhibitory activity of the unknown can be expressed in terms of milligrams of the standard inhibitor preparation per milliliter of solution.

10. Saline Buffer.—This consisted of the borax-borate mixture described by Clark (1928, page 213), containing 0.9 per cent NaCl and adjusted to pH 7.4.

Determinations of pH were made with a glass electrode. The temperature in all experiments was controlled to ± 0.1°C. Merthiolate 1:10,000 or 1:20,000 was used as a preservative in all organic solutions.

EXPERIMENTAL

Identity of Lysin Factor and Serum Protease

Measurements of the pH of optimum activity, pH of maximum stability, and temperature-activity relationships were made in order to characterize the chloroform-activated serum protease and fibrinolysin-activated lysin factor. Under the experimental conditions, both of these proteolytic enzyme preparations were found to be identical in these respects.

1. pH of Optimum Activity.—Preparations of fibrinolysin-activated lysin factor and chloroform-activated serum protease in saline buffer were adjusted to various pH values by the addition of HCl or NaOH, brought to 35°C., and mixed with gelatin at the same pH. The mixtures were immediately placed in Ostwald viscosimeters and the proteolytic activity determined by the

method described previously. The data obtained in these experiments are plotted in Fig. 1.

It is evident from Fig. 1 that the two systems are most active at the same pH, in a rather narrow range between pH 7.2 and 7.6, and both show the same rapid decrease in proteolytic activity as the pH is increased or decreased from this range.

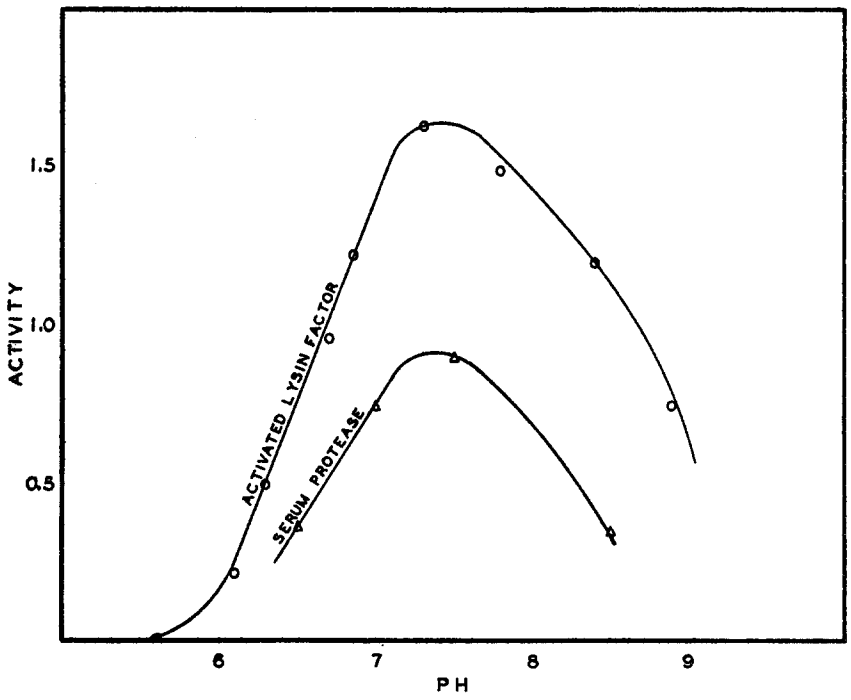

FIG. 1. pH of optimum activity of fibrinolysin-activated lysin factor and chloroform-activated serum protease. Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

2. pH of Maximum Stability.—In determining the pH of maximum stability of the two proteases, solutions of the fibrinolysin-activated lysin factor and chloroform-activated serum protease in saline buffer were adjusted to the desired pH values with HCl or NaOH and incubated at 45°C. for 30 minutes. At the end of this time they were removed, neutralized, adjusted to constant volume, brought to 35°C. as rapidly as possible, and tested for proteolytic activity against gelatin at pH 7.4. 45°C. was chosen as the inactivating temperature because the effects were more rapid than at lower temperatures, permit-

ting more observations to be made in a given time. The values obtained are shown in Fig. 2.

From the observations recorded in Fig. 2 it can be seen that at 45°C. both fibrinolysin-activated lysin factor and chloroform-activated serum protease have their maximum stability in a narrow range between pH 7.0 and 7.4, with rapid loss of activity above and below this pH range.

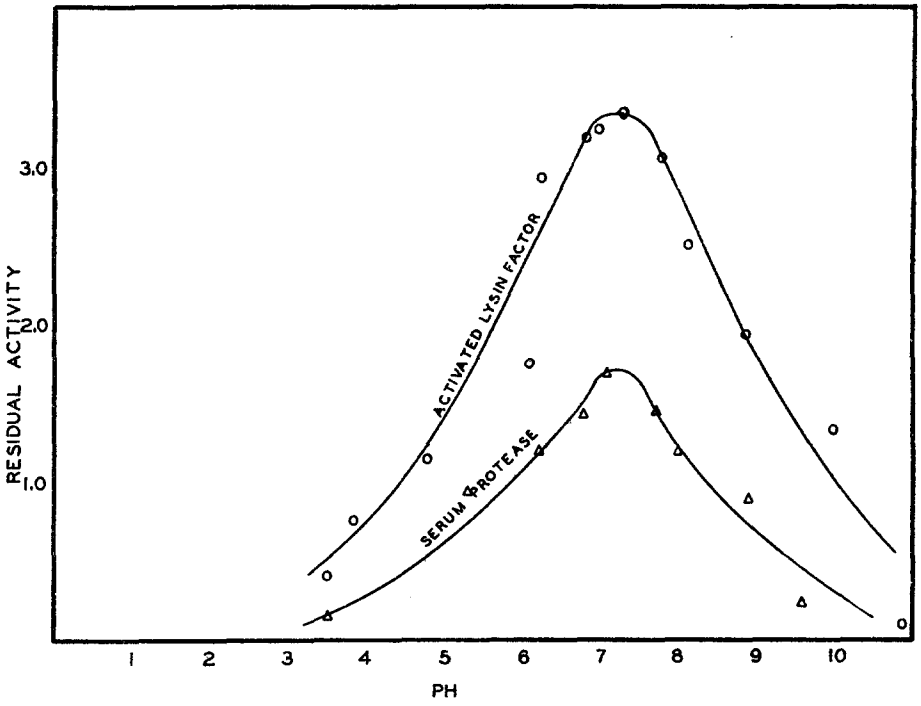

FIG. 2. pH of maximum stability of fibrinolysin-activated lysin factor and chloroform-activated serum protease at 45°C. Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

3. Temperature-Activity Relationships.—An Arrhenius plot of the temperature activity relationships of the two preparations was made. In determining the activity values for the Arrhenius plot, the proteolytic activity of the two preparations at the various temperatures was measured in the manner described, using gelatin at pH 7.4 as substrate. The temperature of each determination was controlled to within ± 0.1°C. In Fig. 3 the logarithms of the activities of the two proteases at the various temperatures have been plotted against the reciprocal of the absolute temperature.

The slopes of the curves for the values obtained with fibrinolysin-activated

lysin factor, as shown in Fig. 3, were calculated by the method of "least squares." Multiplication of arbitrary points on these calculated curves by a constant factor enables one to transpose the lysin factor curves to the experimental values obtained with chloroform-activated serum protease. When this is done, it can be seen that the lysin factor curves fit the serum protease values

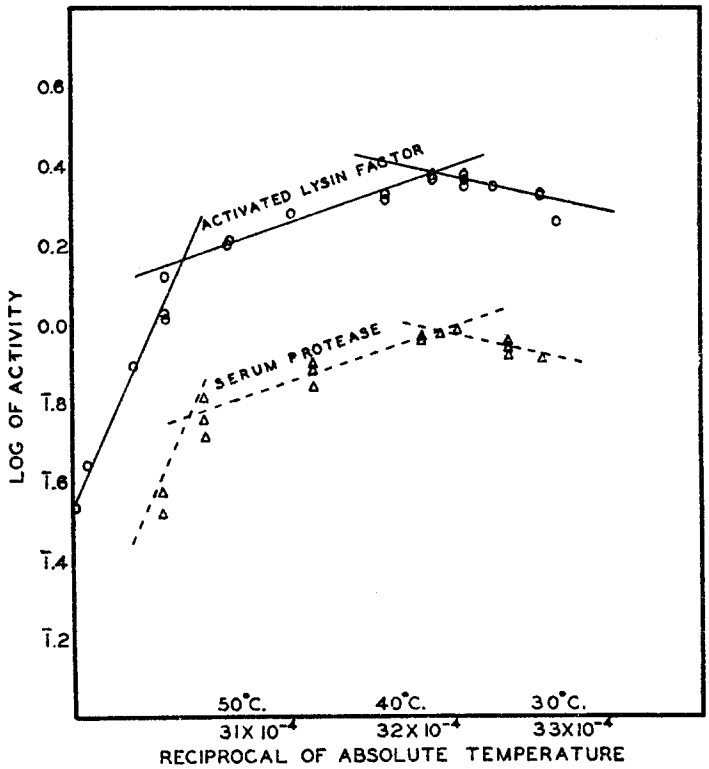

FIG. 3. Arrhenius plot of fibrinolysin-activated lysin factor and chloroform-activated serum protease. Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

quite well, indicating that the Arrhenius plots of the two preparations are identical. This calculation would appear to be valid because the slopes of an Arrhenius plot are independent of the units employed or the concentration of the enzyme, provided the units used are directly proportional to the enzyme concentration.

The Arrhenius plots presented in Fig. 3 indicate that the optimum temperature for the two preparations is about 37°C., as shown by the sharp change in direction of the plots at this point. Above 37°C. the activity decreases, pos-

sibly due to heat denaturation of the enzyme. A marked increase in the rate of inactivation is shown by both preparations as the temperature is raised above 53°C. The exact significance of this second break in the curve is not known; however, Sizer (1944) has recently reported a similar break in an Arrhenius plot of catalase.

It is possible to calculate the energy of activation and energy of inactivation of an enzyme from the slopes of an Arrhenius plot (Sizer, 1943). Calculation of these constants from the calculated slopes of the activated lysin factor plot indicates that the heat of activation is about 14,000 to 16,000 calories. The energy of inactivation in the region between 37° and 53°C. is about 50,000 calories, and in the region above 53°C. the energy of inactivation is about 400,000 calories. Because of inherent errors in the methods used, these figures are only approximations of the true values. For example, the viscosity of gelatin is not a regular function of temperature, nor does the viscosity of gelatin remain constant at higher temperatures in the absence of enzyme. Further, when gelatin is used as substrate, viscosity measurements cannot be made with any accuracy below a temperature of 30–32°C. Despite these sources of error, the energies of activation and inactivation are of the order of magnitude of many enzymes (Sizer, 1943).

The close similarity between fibrinolysin-activated lysin factor and chloroform-activated serum protease in respect to the effects of pH and temperature on their activity leaves little doubt that the two are very similar if not identical. In subsequent portions of the present paper, therefore, the term "serum protease" will be used to designate the proteolytic activity of serum, and unless otherwise specified will indicate fibrinolysin-activated material.

Activation of Serum Protease by Fibrinolysin

A study of the fibrinolysin activation of serum protease is accompanied by certain difficulties. Of these, the most important is that caused by the presence of variable amounts of serum inhibitor in the serum protease preparations, resulting in an apparent alteration in the course of the activation reaction. Furthermore, since no method has been found of destroying or inactivating fibrinolysin without at the same time destroying serum protease, some further activation of serum protease occurs during the period of testing for liberated serum protease activity. For these reasons the values obtained in the present study are only approximations of the true values.

These difficulties were minimized as much as possible by studying the activation of a sample of serum protease which contained only a small amount of serum inhibitor. The effect of continued activation during testing was minimized by activating with dilute solutions of fibrinolysin, so that the further 14-fold dilution of the fibrinolysin which occurs during the determination of proteolytic activity of the mixture would lower the concentration of fibrinolysin below an effective level.

1. Activation of Serum Protease by Fibrinolysin in the Presence of Serum Inhibitor.—Kunitz (1938) has shown that the formation of trypsin from trypsinogen by the mold kinase follows the course of a catalytic unimolecular, or first order reaction, which may be expressed by the equation

$$\log \frac{Ae}{Ae - A} = (KM)t$$

where Ae is the trypsin activity after complete activation, A is the concentration of active trypsin in the activation mixture at any time t, and (KM) is the slope of the straight line obtained when the values of

$$\log \frac{Ae}{Ae - A}$$

are plotted against the values for t.

In testing the activation of serum protease, aliquots of inactive serum protease preparations (lysin factor) were mixed with equal volumes of dilutions of a fibrinolysin concentrate and incubated at 25°C. in a water bath. At intervals, 1 ml. of the mixture was removed and tested for proteolytic activity. Two serum protease preparations were used in this study. Both had the same amount of proteolytic activity when treated with an excess of fibrinolysin; 3.0 proteolytic units per ml. of activation mixture. Preparation 514 contained serum inhibitor equivalent to 0.001 mg. per ml. of the standard trypsin inhibitor preparation, as determined by the procedure described in the section on methods. The second preparation, No. 598, contained inhibitor equivalent to 0.0047 mg. per ml. The data obtained in these experiments were analyzed by means of the equation used by Kunitz (1938) and the results plotted in Fig. 4.

It is evident from Fig. 4 that the activation of serum protease preparation 514, containing the least amount of inhibitor, is a linear function of the time of incubation, indicating a first order reaction (Kunitz, 1938). With the highest dilutions of fibrinolysin, 1:400 and 1:500, the curves deviate somewhat from a linear relationship, probably due to the action of serum inhibitor on the small amount of free enzyme which would be present at any time in the mixture. This conclusion is borne out by the results obtained with the second serum protease preparation, No. 598, which contains about 5 times as much inhibitor as preparation 514. In this case a linear relationship is not obtained, even with the higher concentrations of fibrinolysin.

It is apparent from the above data, therefore, that the kinetics of fibrinolysin activation of serum protease is not that of a first order reaction in the presence of serum inhibitor. However, as the concentration of inhibitor decreases, the kinetics approaches that of a first order reaction, suggesting a catalytic type of

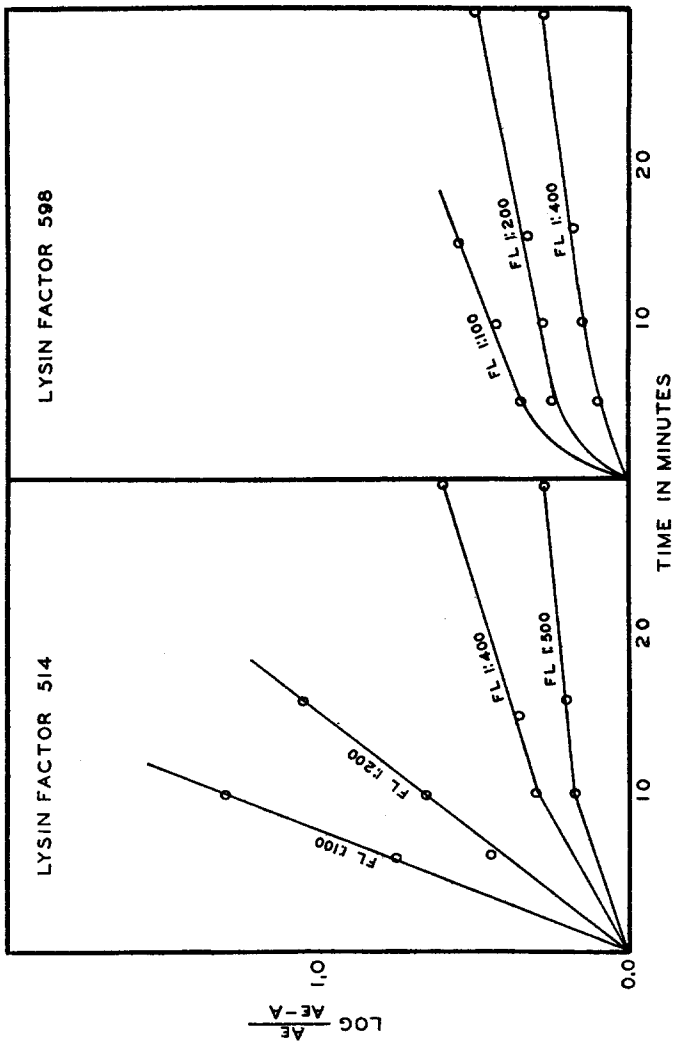

FIG. 4. Rate of activation of serum protease by streptococcal fibrinolysin. The left-hand chart represents the activation of preparation 514, which contained 0.001 mg. inhibitor per ml. The right-hand chart represents the activation of preparation 598, which contained 0.0047 mg. of inhibitor per ml. Both preparations possessed the same amount of proteolytic activity when activated by an excess of fibrinolysin.

activation of the serum protease by fibrinolysin, analogous to the kinase activation of trypsinogen.

2. Effect of Fibrinolysin Concentration on Rate of Activation.—In Fig. 4 the slopes of the lines are proportional to the rate of activation (Kunitz, 1938). In Table I are presented the slopes of the activation curves of serum protease preparation 514 when activated by varying concentrations of fibrinolysin, calculated from the data presented in Fig. 4.

From the calculations shown in Table I it can be seen that the slopes of the activation curves of the serum protease preparation are directly proportional to the concentration of fibrinolysin, except in the case of the more dilute preparations of fibrinolysin. The lack of proportionality in these cases is probably due to the presence of serum inhibitor in the sample, as has been discussed above. This is borne out by the fact that in the presence of higher concentra-

TABLE I

Effect of Fibrinolysin Concentration on the Rate of Activation of Serum Protease

Fibrinolysin concentration	Slope of activation curve*
1:100	0.065
1:200	0.0325
1:400	0.015
1:500	0.006

* The slope of the curve is a measure of the rate of activation.

tions of serum inhibitor than are present in preparation 514 (*e.g.* preparation 598), the rates of activation are not proportional to the fibrinolysin concentration, even when higher concentrations of fibrinolysin are used. On the other hand, with low concentrations of serum inhibitor the activation rates are more nearly proportional to the concentration of fibrinolysin. It appears highly probable, therefore, that in the absence of serum inhibitor, the rate of activation of serum protease by fibrinolysin would be directly proportional to the concentration of fibrinolysin, suggesting an enzymatic type of activation.

3. Temperature Coefficient of Activation.—The temperature coefficient of the activation reaction was determined by mixing aliquots of serum protease preparation 514 with equal volumes of a 1:200 dilution of fibrinolysin and incubating the mixtures at 25°C. and 35°C. At intervals, 1 ml. of the mixture was removed and tested for proteolytic activity. The results obtained are plotted in Fig. 5 in the same manner as in the previous experiment, using the equation of Kunitz (1938).

Calculation of the slopes of the curves in Fig. 5 reveals that an increase in temperature of 10°C. between 25° and 35°C. results in an increase of 1.76-fold in the rate of activation, indicating that the temperature coefficient of the reaction between these temperatures is about 1.8.

The data obtained in the above experiments are compatible with the hypothesis that the activation of serum protease by fibrinolysin is an enzyme-catalyzed reaction. The kinatics of activation is those of a first order reaction with the rate of activation directly proportional to the fibrinolysin concentration and the temperature coefficient of the reaction about 1.8 between 25° and 35°C. In the presence of serum inhibitor, however, the reaction appears to be complicated by combination of active enzyme with inhibitor, which causes the kinetics to differ from that of a first order reaction.

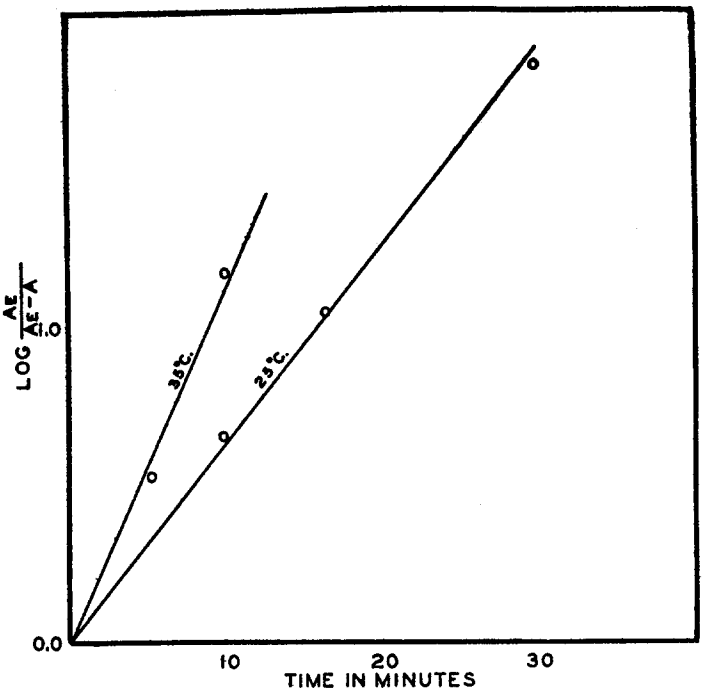

FIG. 5. The effect of temperature on the rate of activation of serum protease by streptococcal fibrinolysin.

Interaction of Serum Protease and Inhibitors

Following the demonstration that the activation of serum protease is an enzymatic type of reaction, it became of interest to determine the mechanism of activation. Mention has been made in the introduction to this paper of the hypothesis that serum protease is normally inactive in serum because of the presence of serum inhibitor.

1. Nature of Serum Protease Inhibition by Crystalline Trypsin Inhibitor and Serum Inhibitor.—Northrop and his coworkers have shown that both pancreatic trypsin inhibitor and serum inhibitor combine with trypsin, and that the kinetics of the reaction indicate the formation of an equimolar, dis-

sociable, inhibitor-enzyme compound (Northrop, 1939; Kunitz and Northrop, 1936; Hussey and Northrop, 1923). In order to determine whether or not the inhibition of serum protease is of similar nature, the inhibition of activated serum protease by crystalline pancreatic trypsin inhibitor and by serum inhibitor was compared with the effect of these agents on crystalline trypsin.

Fibrinolysin-activated serum protease was mixed with equal volumes of crystalline trypsin inhibitor solution of varying concentrations. The mixtures were incubated at 35°C. for 10 minutes to allow combination, and 1 ml. of the mixture was then tested for residual proteolytic activity against gelatin. A trypsin solution containing 0.01 mg. per ml. of the crystalline trypsin preparation was tested with inhibitor solutions in the same way. This concentration of trypsin was chosen because it possessed about the same proteolytic activity under the conditions of the present experiment as the activated serum protease preparations. The results of these experiments are plotted in Fig. 6. The curve drawn through the values obtained for the inhibition of trypsin was recalculated from the data of Northrop (1939, p. 11) on the inhibition of trypsin by trypsin inhibitor.

It is evident from the observations shown in Fig. 6 that the reaction between serum protease and crystalline trypsin inhibitor differs markedly from the reaction of trypsin with the inhibitor. Not only is the inhibitor much less active against serum protease than against trypsin, but the shapes of the two inhibition curves are so different as to suggest a marked qualitative difference in the inhibition of the two enzymes by crystalline trypsin inhibitor.

The experiment described above was repeated except for the substitution of serum inhibitor for the crystalline trypsin inhibitor, although available evidence suggests they are the same substance (Schmitz, 1938; Grob, 1943). The results are plotted in Fig. 7, with the curve drawn through the values obtained with trypsin recalculated from the data of Hussey and Northrop (1923) on the inhibition of trypsin by serum.

The data recorded in Fig. 7 indicate the same qualitative and quantitative differences in the inhibition of serum protease and trypsin by serum inhibitor as were found in the experiments employing the crystalline trypsin inhibitor from the pancreas.

Experiments have been carried out with chloroform-activated serum protease using the two inhibitors. The chloroform-activated protease behaved in the same manner as the fibrinolysin-activated material.

It is apparent, therefore, that although both crystalline trypsin inhibitor from the pancreas and serum inhibitor affect the activity of serum protease, the reaction is not the same as when these inhibitors act on crystalline trypsin.

2. Effect of Fibrinolysin on Serum Protease Inhibition.—It has been assumed by several investigators that serum protease is normally inactive because of the presence of serum inhibitor (Delezene and Pozerski, 1903; Yamakawa, 1918; Schmitz, 1937; Jühling and Wöhlisch, 1938). If this were correct it should be

possible to demonstrate an action of fibrinolysin on either the isolated inhibitor or on the inhibitor-enzyme complex.

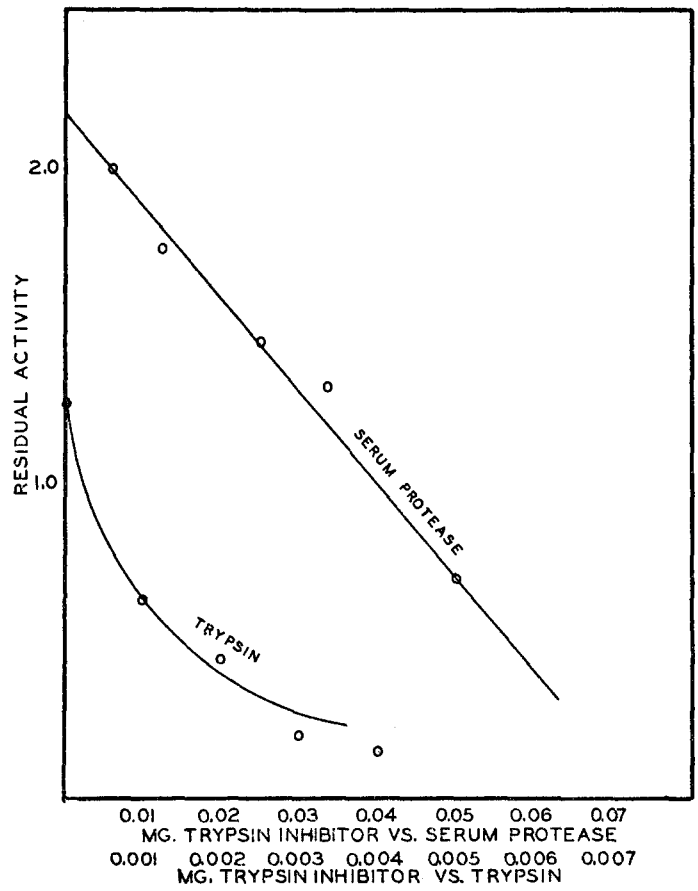

FIG. 6. Inhibition of serum protease and crystalline trypsin by crystalline trypsin inhibitor. The concentrations of inhibitor used with trypsin are only $\frac{1}{10}$ those used to inhibit serum protease. The curve drawn through the values for crystalline trypsin was recalculated from data of Northrop (1939) and superimposed on the experimental points. Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

A sample of lysin factor was activated by adding a small amount of fibrinolysin solution and allowing the mixture to stand at room temperature for several hours. When activation was complete, the activated protease was salted out by the addition of 1/2 volume of saturated ammonium sulfate. The precipitate was collected by centrifugation, washed several times with 1/3 saturated ammonium sulfate solution, and reprecipitated. It was then dialyzed sulfate-free and made up to the original volume

in saline buffer. This procedure was employed in order to reduce the residual fibrinolysin concentration of the solution as much as possible.

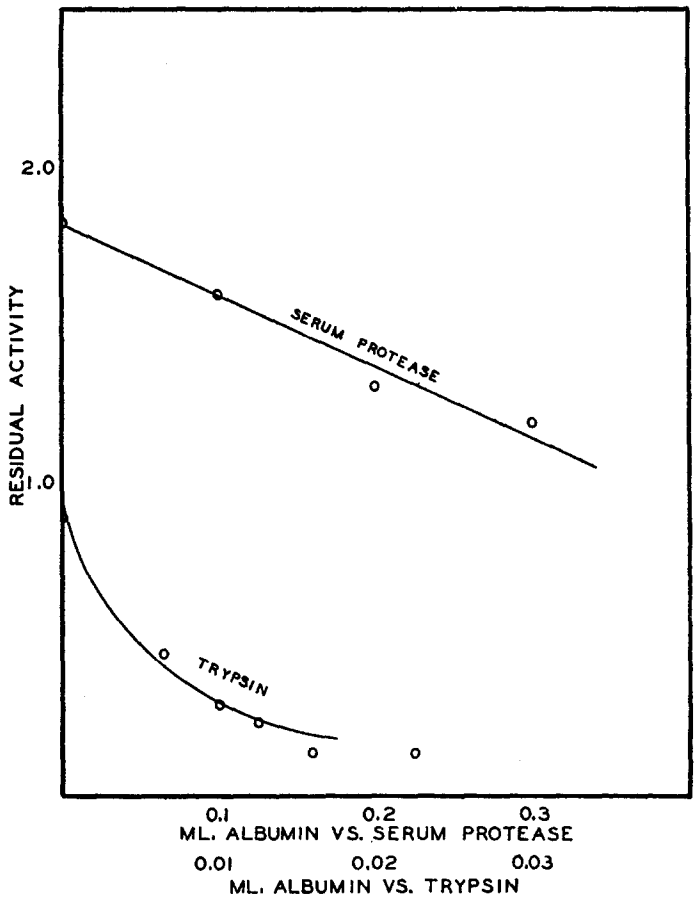

FIG. 7. Inhibition of serum protease and crystalline trypsin by serum inhibitor. The amounts of serum inhibitor (albumin solution) used to inhibit crystalline trypsin are $\frac{1}{10}$ those used to inhibit serum protease. The curve drawn through the values obtained for crystalline trypsin was recalculated from data of Hussey and Northrop (1923) on the inhibition of trypsin by serum, and was superimposed on the experimental points. Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

In testing the effect of fibrinolysin on the inhibition of the activated protease, a series of tubes was set up, each containing 0.5 ml. of activated protease, 0.5 ml. of a solution of crystalline trypsin inhibitor, and 0.1 ml. of dilutions of a fibrinolysin concentrate. The mixtures were incubated at 35°C. for 10 minutes and tested for residual proteolytic activity. Control tubes contained respectively serum protease alone, serum protease with inhibitor, and serum protease with fibrinolysin. In order

to avoid an excess of inhibitor, the amount of crystalline inhibitor used was insufficient to inhibit the protease completely. The results of this experiment are shown in Table II.

As shown in Table II, under the experimental conditions employed fibrinolysin did not increase the activity of the partially inhibited protease. The lack of an activating effect of fibrinolysin does not appear to be due to insufficient fibrinolysin since it was observed repeatedly that the same fibrinolysin preparation was capable of activating completely within 10 minutes all lysin factor preparations with which it was tested.

TABLE II

Effect of Fibrinolysin on the Inhibition of Serum Protease by Crystalline Pancreatic Trypsin Inhibitor

Inhibitor concentration	Residual activity after incubation in proteolytic units			
	Concentration of fibrinolysin			
	0	1:10	1:100	1:1000
mg. per ml.				
0	1.66	1.20	—	1.54
0.02	1.38	0.72	0.98	1.20

TABLE III

Effect of Fibrinolysin on Inhibition of Serum Protease by Serum Inhibitor

Inhibitor concentration	Residual activity after incubation in proteolytic units			
	Concentration of fibrinolysin			
	0	1:10	1:100	1:1000
ml. of albumin				
0	1.66	1.20	1.42	1.60
0.2	1.3	1.02	1.37	1.45
0.5	0.9	0.72	0.77	0.78

It can also be seen from the data shown in Table II that the fibrinolysin solutions, rather than increasing the activity of the partially inhibited protease, actually caused a decrease in the residual activity, suggesting the presence of an inhibitor of proteases in the fibrinolysin solution itself. Such an inhibitor of proteases could be demonstrated in fibrinolysin preparations by adding them to trypsin solutions.

The experiment described above was repeated except for the substitution of serum inhibitor for crystalline trypsin inhibitor. The results of this experiment are shown in Table III.

As in the previous experiment, the data presented in Table III indicate that fibrinolysin does not increase the activity of the partially inhibited protease.

Likewise, it appears that the fibrinolysin solution itself caused some inhibition of the protease.

A number of variations of these experiments has been carried out. Incubation of fibrinolysin and inhibitor before addition of the protease was found to be without effect on the degree of inhibition. Furthermore, incubation of the protease and inhibitor, or protease and fibrinolysin before the addition of the third reagent was likewise without effect on the degree of inhibition. Chloroform-activated protease behaved in the same way as the fibrinolysin-activated material, that is, fibrinolysin did not decrease the amount of inhibition caused by either inhibitor.

It has also been found that the inhibition of crystalline trypsin by either serum or pancreatic inhibitor is not influenced by the presence of fibrinolysin in the reaction mixture, although again the fibrinolysin solutions inhibit the proteolytic activity of the trypsin to some extent.

It appears, therefore, that streptococcal fibrinolysin is entirely without effect on the inhibition of serum protease by serum or pancreatic inhibitors. Furthermore, since fibrinolysin is capable of activating serum protease from its normal inactive state in serum, it follows that this inactive state of serum protease is not due to combination of the protease with serum inhibitor, but to some other mechanism.

Substrate Specificity of Serum Protease and Trypsin

The reactions of serum protease and trypsin with protease inhibitors, together with the characteristics of serum protease as described in a previous section of the present paper, suggest that serum protease and trypsin are dissimilar enzymes.

Northrop (1939, p. 76) has shown that when an enzyme is allowed to act on a protein substrate until hydrolysis has virtually ceased, addition of a second quantity of the same enzyme does not result in further hydrolysis. On the other hand, if a different enzyme is added, further hydrolysis of the substrate takes place. This phenomenon is probably an expression of the different peptide bond specificities of the two enzymes, since, as Bergmann and his coworkers (1941, 1942) have shown, each proteolytic enzyme is characterized by the ability to hydrolyze certain specific peptide linkages. In Northrop's experiment, therefore, once all of the bonds susceptible to one enzyme are split, no further action can be produced by this enzyme although further hydrolysis will occur on the addition of an enzyme having different specificities.

In order to determine whether or not the serum enzyme and trypsin attack the same linkages of the substrate molecule, 20 ml. aliquots of a 5 per cent casein solution were mixed with 5 ml. portions of a solution of either serum protease or trypsin. At intervals, 1 ml. portions of the mixtures were removed, precipitated with trichloracetic acid, and the acid-soluble tyrosine in the filtrate determined by a method outlined in a previous paper (Christensen, 1945). The following day, when the rate of acid-

soluble tyrosine liberation had dropped to nearly zero, the reaction mixtures were divided into three portions. In one portion the rate of acid-soluble tyrosine liberation was followed without the addition of other reagents; to the second portion was added a second quantity of the enzyme first used; to the third portion of the reaction mixture containing trypsin, serum protease was added, and to the reaction mixture containing serum protease, trypsin was added. The results obtained in this experiment are plotted in Fig. 8, where the milligrams of acid-soluble tyrosine liberated per milliliter of reaction mixture are plotted against the incubation time.

It is evident from the data presented in Fig. 8 that serum protease rather quickly liberates from casein the majority of the acid-soluble tyrosine which it is capable of liberating and addition of fresh enzyme does not result in any further increase. However, if trypsin is added to the serum protease digest, a prompt and marked rise in acid-soluble tyrosine liberation occurs, indicating that linkages are still available for the action of trypsin after all those susceptible to serum protease have been split. On the other hand, if casein is first digested by trypsin, addition of serum protease to the digest mixture does not result in further hydrolysis of the casein, although the increase in acid-soluble tyrosine on addition of more trypsin indicates that hydrolysis by trypsin is not yet complete. This might be taken as an indication that trypsin is able to hydrolyze not only the linkages split by serum protease, but others as well. In any case, it is evident that trypsin is able to continue hydrolysis of casein after hydrolysis by serum protease has gone to completion.

DISCUSSION AND SUMMARY

The observations recorded in the present paper indicate that the lysin factor described by Milstone (1941) and the chloroform-activated serum protease are one and the same proteolytic enzyme, differing only in their mode of activation. The serum protease is most stable at a pH near 7.2, and its pH of optimum activity is also in this region. The pH of optimum activity may be not so much a reflection of the pH at which the individual molecules of the enzyme are most active, but instead be related to the marked instability of the enzyme above and below this pH range. The Arrhenius plot of the temperature-activity characteristics of the enzyme indicates that the optimum temperature for activity is about 37°C. Above this temperature activity falls off rapidly, probably due to heat inactivation. A second sharp break in the Arrhenius plot occurs at a temperature of about 53°C., above which point the activity of the enzyme decreases with great rapidity. This type of two-phase inactivation curve for an enzyme has recently been discussed by Sizer (1944) in relation to a similar phenomenon which occurs with catalase. The energies of activation and inactivation of the enzyme, as calculated from the slopes of the Arrhenius plot, are only approximations because of certain errors inherent in the methods used. However, the values obtained, 16,000 calories and 50,000 calories respectively, are of the order of magnitude characteristic of most enzymes. The energy of inactivation above 53°C., about 400,000 calories,

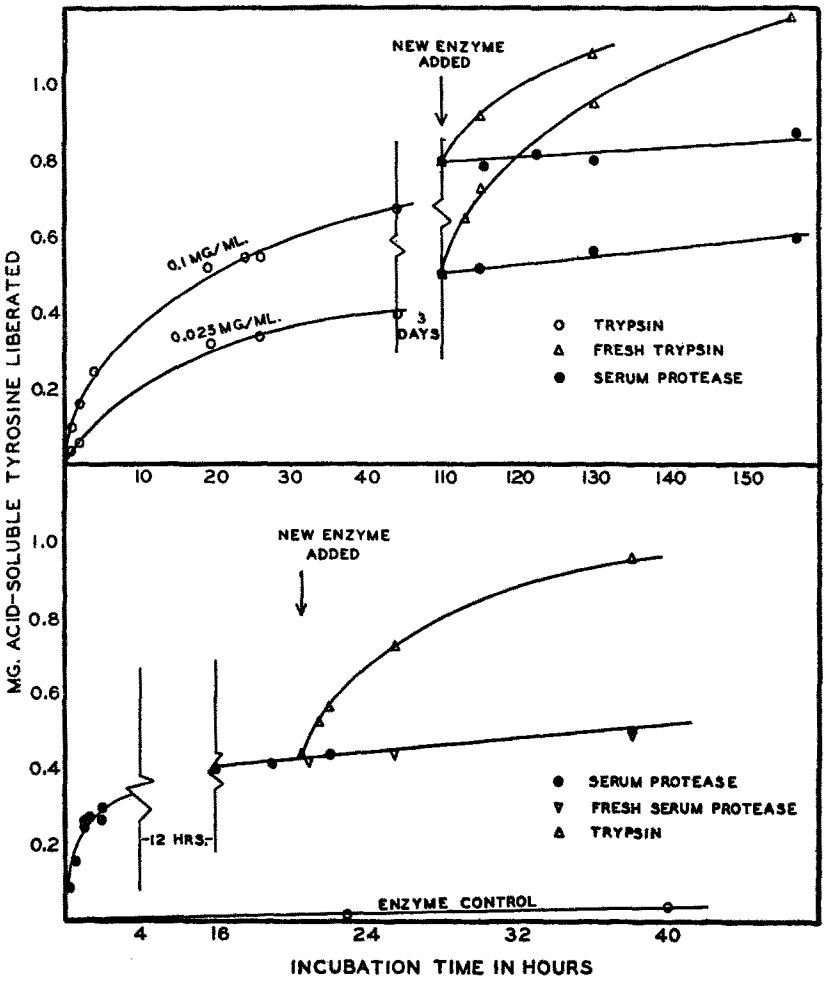

FIG. 8. Combined action of serum protease and trypsin on casein. The upper portion of the figure represents the hydrolysis of casein by trypsin, followed by the addition of fresh trypsin solution (containing 0.5 mg. of the dry material per ml.) and serum protease solution (containing about 2.8 proteolytic units per ml.). The lower portion of the figure represents the hydrolysis of casein by serum protease solution (2.8 proteolytic units per ml.), followed by the addition of fresh serum protease solution and by trypsin solution (0.5 mg. per ml.). Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

is higher than the values recorded by Sizer (1943) for most other enzymes. The actual value is subject to some error, but the order of magnitude for the inactivation energy of serum protease above 53°C. agrees with the higher value found for catalase by Sizer (1944), namely 255,000 calories.

It seems apparent that serum protease is not trypsin nor is it similar to trypsin in its properties, as has been implied previously (Schmitz, 1936, 1937; Iyengar, 1942; Ferguson, 1939, 1940 *a*, *b*; Tagnon, 1942 *a*, *b*). Trypsin, in contrast to serum protease, does not exhibit as sharp a zone of maximal activity in the neighborhood of pH 7.4, with the marked drop in activity above and below this point that is shown by serum protease. Crystalline trypsin is most stable at a pH of about 3, but crude preparations are stable over a range of about pH 6-8. Since the serum protease preparation is quite crude, it is not possible to state that the two enzymes differ in this respect.

Other evidence of the non-identity of the two enzymes, trypsin and serum protease, is brought out by the difference in behavior toward protease inhibitors. Hussey and Northrop (1923), Kunitz and Northrop (1936), and Grob (1943) have shown that the kinetics of inhibition by either serum or pancreatic inhibitor indicates the reaction to be due to the formation of a dissociable, equimolar compound between enzyme and inhibitor. The inhibition of serum protease by these two inhibitors, however, requires much more of either inhibitor in order to inhibit the same amount of proteolytic activity than in the case of trypsin. In addition to this marked quantitative difference, the shape of the inhibition curves of the two enzymes in the presence of either inhibitor differs so markedly as to indicate the possibility of a different mechanism of inhibition. Several explanations might be advanced for these differences in the inhibition of the two enzymes. It is possible that the inhibition of serum protease is not an equimolar reaction, but that several molecules of inhibitor must combine with the enzyme before inhibition is complete. It is also possible that trypsin is more actively proteolytic per molecule than is serum protease, or it is possible that the dissociation of the serum protease-inhibitor complex differs greatly from that of the trypsin-inhibitor complex.

The identical behavior of both the serum and pancreatic inhibitors toward both trypsin and serum protease furnishes additional evidence that the two inhibitors are very similar if not identical, as postulated by Schmitz (1938).

Further evidence as to the non-identity of the two enzymes is shown in the study of their combined effect on a single substrate. The data indicate quite clearly that trypsin is able to continue the hydrolysis of casein after hydrolysis by serum protease is practically complete. In the light of the work of Bergmann (1941, 1942) and his coworkers on the peptide bond specificity of proteases, this may be interpreted as indicating that serum protease and trypsin split different linkages in the substrate molecule. When the converse of this experiment was carried out, that is, when trypsin was allowed to act first, followed by serum protease, no further hydrolysis took place, even though the action of trypsin was not complete, as indicated by further hydrolysis on the addition of more trypsin. In explaining these findings it may be suggested that the linkages split by serum protease are among those split by trypsin and

that the latter enzyme is able to split additional linkages. It is also possible that the linkages split by serum protease are contained in the acid-soluble fragments of trypsin digestion, and under the conditions of the experiment it is not possible to detect the hydrolysis of these bonds by serum protease. In any event, it is apparent that trypsin is able to split linkages in casein which serum protease cannot.

It has not been possible in any experiment so far devised to demonstrate that fibrinolysin affects in any way the inhibition of serum protease or of trypsin by either serum or pancreatic inhibitors.[4] It appears, therefore, that the normal inactive state of serum protease in the serum is not due to the inhibitor also present in the serum, but involves some other mechanism. It is interesting to note that prior to the studies of Kunitz and Northrop the inactivity of pancreatic extracts was explained on the basis that the proteases were combined with the inhibitor also present in the same extracts (Northrop, 1939). Kunitz and Northrop (1936) demonstrated that these enzymes are inactive because they occur in a precursor or zymogen state from which they can be activated by certain specific activators (kinases) and in some cases by non-specific agents such as magnesium and ammonium sulfate. Following activation from the precursor state, the active enzyme may then combine with inhibitor with resulting loss of activity.

While it has not been possible to demonstrate an action of fibrinolysin on serum protease inhibited by either serum or pancreatic trypsin inhibitors, it has been shown that the activation of serum protease from its normal state in serum by fibrinolysin is apparently a catalytic reaction. The kinetics of this reaction, in the absence of large amounts of serum inhibitor, indicates that it is enzymatic in character, as is the kinase activation of the pancreatic proteases.

[4] Mirsky (1944) has recently reported inhibition of the fibrinolytic activity of streptococcal cultures by addition of crystalline trypsin inhibitor of Kunitz and Northrop (1936) and the inhibitor extracted from soybeans by Ham and Sandstedt (1944). Mirsky suggests that inhibition is due to inactivation of fibrinolysin by inhibitors and that fibrinolysin is a protease related to trypsin. The apparent contradiction between the results presented in the present paper and those obtained by Mirsky would appear to be based on differences in the amounts of inhibitor used. A satisfactory method for the measurement of the small amount of proteolytic activity in unconcentrated serum has not been available to us, but based on estimates of the proteolytic activity of unconcentrated serum, and the amount of crystalline trypsin inhibitor necessary to inhibit the serum protease concentrates, it appears that Mirsky used levels of trypsin inhibitor 100–500 times as high as would be necessary to completely inhibit all of the serum protease activity in his fibrinogen preparations. Thus, even though fibrinolysin was added to the mixture, the tremendous excess of inhibitor would result in inactivation of serum protease as rapidly as it was activated. The results obtained by Mirsky, therefore, are probably due to inhibition of the activated protease by an excess of inhibitor rather than to inhibition of fibrinolysin.

The rate of activation is directly proportional to the fibrinolysin concentration and the activation has a temperature coefficient of about 1.8. Following activation by fibrinolysin, the active protease may be inhibited by serum or pancreatic trypsin inhibitor, but as noted above, this inhibition cannot be influenced by fibrinolysin. In the presence of serum inhibitor, the kinetics of serum protease activation is complicated by combination of the activated enzyme with inhibitor, causing the course of the reaction to deviate from that of a first order reaction. Northrop (1939) has shown that the kinetics of activation of pepsinogen and trypsinogen also shows a deviation from the theoretical in the presence of inhibitors of these enzymes.

Serum protease may also be activated by chloroform and other non-specific agents. The mechanism of activation by these agents requires further study. It is possible that one of two or more mechanisms is involved. Bodine and his coworkers (1937, 1938, 1943, 1944) have shown that treatment with certain denaturing agents, such as chloroform or certain detergents, activates the protyrosinase of grasshopper eggs. Activation apparently involves slight denaturation of the proenzyme protein. On the other hand, the function of chloroform in the activation of serum protease may be the removal of serum inhibitor, allowing spontaneous activation of the protease, analogous to the spontaneous activation of trypsinogen which occurs in the absence of trypsin inhibitor. Certain evidence supporting this latter hypothesis is available in the case of serum protease. Teale and Bach (1919) have shown that serum inhibitor can be removed from serum by organic solvents only when coagulation of protein occurs. In this laboratory it has been noted that solutions of inactive serum protease (lysin factor) which contain little or no serum inhibitor may become active spontaneously on standing for several weeks in the refrigerator (Christensen, 1945). It is possible, therefore, that chloroform treatment of serum results in the removal of serum inhibitor by producing protein denaturation. Upon removal of the inhibitor, spontaneous activation of the serum protease may occur. This spontaneous activation may be autocatalytic in nature. The proteolytic enzyme precursors pepsinogen and trypsinogen are autocatalytically activated with great rapidity, which is apparently not true of serum protease. On the other hand, chymotrypsinogen shows some spontaneous activation, but the process is much slower than with pepsinogen and trypsinogen (Kunitz and Northrop, 1935).

The data obtained in the present experiments are compatible with the hypothesis that the proteolytic enzyme system of serum is analogous to the proteolytic enzyme systems of the pancreas, trypsin and chymotrypsin. The serum enzyme occurs normally in serum and plasma in an inactive precursor state, similar to trypsinogen and chymotrypsinogen. Activation from this precursor state can be accomplished by treatment with streptococcal fibrinolysin, which acts in a manner analogous to enterokinase or the

mold kinase of Kunitz (1938). Activation may also be produced by other means, the mechanism of which is not yet understood, but which may involve autocatalytic activation. Following activation, the active enzyme, in common with trypsin, may be inhibited by crystalline pancreatic trypsin inhibitor or an inhibitor present in serum.

Study of the properties of serum protease suggests that a revised terminology should be introduced in order to describe it more adequately. Terms such as "serum trypsin" and "serum tryptase" are unsuitable because they imply a relationship between the serum protease and trypsin. The data recorded in the present paper indicate that these enzymes are dissimilar. The older terms "fibrinolysin" and "serum fibrinolysin" are also unsuitable because the enzyme is active against proteins other than fibrin. "Serum protease" is not completely satisfactory because it is binomial and also because the term "serum proteasogen" to indicate the zymogen form is cumbersome. Since the proteolytic enzyme of serum appears to be analogous to that of certain of the pancreatic proteases, a nomenclature based on the principles employed in designating the latter enzymes is suggested. Under this scheme the activated enzyme may be termed "plasmin" in comformity with common usage for proteases, where the prefix indicates the source of the enzyme, followed by -in, as with "bromelin," "ficin," and "papain." The inactive enzyme as it occurs in serum and plasma may be designated as "plasminogen" to indicate its source, the plasma, and also to indicate that it is in an inactive, precursor state. Streptococcal fibrinolysin, also a misnomer in the light of present knowledge, may be termed "streptokinase," analogous to "enterokinase" or "mold kinase." The term "plasmin" has been used in the past to designate a fraction of blood obtained by a special salting-out procedure. This usage, however, has become obsolete and the possibility of confusion with the proteolytic enzyme system is remote.

CONCLUSIONS

1. Fibrinolysin-activated lysin factor and chloroform-activated serum protease of serum and plasma are one and the same enzyme, differing only in their mode of activation.

2. The enzyme as it normally occurs in serum or plasma is not inactive because of combination with serum inhibitor. It is present as an inactive precursor or zymogen and may be activated from this state by streptococcal fibrinolysin.

3. The activation of serum protease by streptococcal fibrinolysin is a catalytic reaction, analogous to the kinase activation of trypsinogen by enterokinase. Treatment of serum or plasma with chloroform apparently results in removal of serum inhibitor which may allow autocatalytic activation of the serum protease.

4. The serum enzyme differs from trypsin in its pH of optimum activity,

in its reactions with specific protease inhibitors, and in its action on casein.

5. A revised nomenclature for the serum enzyme system is suggested which more accurately describes its properties than the terms in current use.

We are grateful to Dr. J. H. Northrop for suggestions during the course of this work.

We wish to acknowledge the invaluable technical assistance of Mrs. J. Fuld throughout this study.

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