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I hereby recommend that the thesis prepared under my supervision by Sanford M. Birnbaum

entitled A Study of the Enzymatic Activity of Streptokinase

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by: Melvin A Logan

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A STUDY OF THE ENZYMATIC ACTIVITY OF STREPTOKINASE

A dissertation submitted to the

Graduate School of Arts and Sciences
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

1950

by

Sanford M. Birnbaum

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In 1933 Tillet and Garner (1) discovered that broth filtrates of hemolytic streptococcal cultures are capable of rapidly liquefying normal human fibrin clots. Of twenty-eight strains of hemolytic streptococci of human origin tested all had this power whereas only three of eighteen strains isolated from lower animals lysed human fibrin.

None of these filtrates were capable of lysing rabbit fibrin clots unless the clot was formed by the action of human thrombin. This, coupled with evidence that lysis of fibrin was proteolytic, led Tillet and Garner to the conclusion that the active filtrate component, which they called fibrinolysin, was a remarkably specific proteolytic enzyme acting only on the product formed by the action of human thrombin on fibrinogen.

Many papers followed immediately, mostly of the survey type. The technique used in all these studies was simply the addition of filtrate to a fibrinogen solution or to oxalated whole plasma and clotting by the addition either of a thrombin preparation or a calcium chloride solution. The time interval between the formation and complete liquefaction of the clot was considered inversely

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proportional to the fibrinolysin concentration. The results of most of this work are difficult to interpret in the light of present knowledge but in general the original findings of Tillet and Garner were borne out. A review by Tillet (2) summarized the work on the subject through 1938.

One paper published by Madison (3) in 1934 is particularly interesting. He was able to show that several fibrinolysins produced by streptococci isolated from lower animals were highly active against fibrin clots of that species and inactive against all others. This work has been largely neglected since, although it would seem that the application of more precise techniques and modern knowledge to the problem would be very interesting.

In 1940, Milstone (4) made the first important step toward elucidating the mechanism of fibrinolysis. Preliminary to a search for 'non-specific' factors in blood that accounted for the resistance to lysis shown by rabbit clots and by some human clots he exhaustively purified his test materials, fibrinogen, and thrombin. The clots formed from these purified materials failed to lyse even in the presence of concentrated filtrates. Milstone immediately tried adding different fractions of human serum to the system. He discovered a substance 'lytic factor' concentrated in the euglobulin fraction which in the presence of

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streptococcal filtrates rapidly lysed the purified fibrin. Rabbit fibrinogen clotted by rabbit thrombin also lysed in the presence of the two substances. Thus the picture in early 1940 was as follows:

$$\text{Fibrin} \xrightarrow[\text{lytic factor}]{\text{fibrinolysin +}} \text{soluble split products}$$

Christensen (5) in 1944 concentrated both fibrinolysin and the lytic factor. He was able to show that a mixture of these two substances was proteolytic against casein, hemoglobin, and gelatin as well as fibrin. He postulated that fibrinolysin acted catalytically on the lytic factor to produce an active proteolytic enxyme with the following evidence.

1) No fibrinolysin solution shows proteolytic activity unless lytic factor is present.

2) Lytic factor may develop proteolytic activity spontaneously on standing or after treatment with chloroform.

3) The total proteolytic activity developed is proportional to the amount of lytic factor present and, except in very dilute solutions, independent of the amount of fibrinolysin added.

Christensen and LacLeod (6) confirmed this postulate by showing that:

1) Serum protease (chloroform activated) and lytic

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factor (fibrinolysin activated) are identical with regard to optimum pH, pH of maximum stability, and temperature activity relationships.

2) The activation of inhibitor-free lytic factor by fibrinolysin is a reaction of the first order with the rate of activation proportional to the fibrinolysin concentration.

Due to the obvious parallel between the activation of trypsinogen by enterokinase and the activation of lytic factor by fibrinolysin it was believed that there may be some identity involved. However, Christensen and MacLeod showed that trypsin further hydrolyzed casein after exhaustive hydrolysis by lytic factor indicating the non-identity of these enzymes. Kaplan (7) showed that the activators of trypsinogen and lytic factor were not interchangeable and that fibrinolysin inhibitor from the blood of streptococcus infected patients was inactive against enterokinase.

Due for the most part to the initial erroneous assumption that the streptococcal filtrates were acting directly on fibrin, there has been much confusion of nomenclature in this field. The following table lists the most widely accepted terms in use today. These terms will be

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used throughout this paper.

Original term	Accepted
Fibrinolysin	Streptokinase
Lytic factor	Plasminogen
Activated lytic factor	Plasmin
Anti-fibrinolysin	Anti-streptokinase
Serum anti-protease	Anti-plasmin

The system, in summary, as presently accepted is as follows:

$$\text{Plasminogen} \xrightarrow[\text{or chloroform}]{\text{streptokinase}} \text{plasmin}$$

$$\text{Protein substrate} \xrightarrow{\text{plasmin}} \text{protein split products.}$$

A recent paper by Cliffton and Downie (39) shows the presence of a plasminogen activatable by streptokinase in the blood of rhesus monkeys as well as in humans. Their demonstration of the absence of this substance in dogs, rabbits, guinea pigs, swine, and goats is consistent with earlier results of other investigators showing the resistance of the fibrin clots of lower animals to streptococcal fibrinolysis.

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Statement of Problem

It was proposed to study from as many points of view as possible the activation of human plasminogen by streptokinase prepared from a culture filtrate of a 'human C' strain of β hemolytic streptococci. The establishment of various physical constants for the reaction and the description of certain of the chemical properties of streptokinase may prove to be a useful basis for comparison with similar systems obtained from different sources.

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PREPARATIONS

Streptokinase

Organism. The culture used for the production of streptokinase was a 'human C' strain of β Streptococcus hemolyticus H46A supplied through the kindness of Dr. L. R. Christensen. It was received in lyophilized form and maintained in this laboratory on the stock culture medium of Boyd, Logan, and Tytell (8). No detectable loss of streptokinase synthesizing ability was noted after many subcultures.

Media and production. The medium used for the production of streptokinase was a 2% proteose peptone broth fortified with beef heart extract. Growth was luxuriant and the rate of glucose utilization was maintained at a high level for the duration of the experiment.

The figures given here are for the preparation of a five liter batch of medium, the largest amount conveniently handled here.

Proteose peptone	100	g.
NaCl	10	g.
$Na_2HPO_4 \cdot 12H_2O$	6.25	g.
Phenol red (0.04% soln.)	25	ml.
Water	1000	ml.

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This solution is made slightly alkaline to phenol red and heated to boiling for 10 minutes. It is then filtered clear with the aid of a little supercel and the pH is adjusted to 7.6. The volume is brought to 4200 ml. in a 6 liter florence flask. The flask is plugged with a cotton plug through which passes a burette tip connected to a short piece of rubber tubing. The medium is then autoclaved for 40 minutes at 15 lbs. pressure.

After cooling the following components are added:

$NaHCO_3$, crystals (sterilized in a sealed tube)	10 g.
50% glucose solution	20 ml.

Beef heart extract*, sterilized by Berkfeld filtration, to a concentration of 0.2% beef heart solids (usually about 350 ml.).

The prepared medium is inoculated with 5 ml. of an eight hours culture of Streptococcus hemolyticus and is incubated over night (16 hours) at 37° C.

The following morning the culture is neutralized by the addition of 5 N NaOH, and a sterile solution containing 160-170 grams of glucose is added. A burette is fixed in place on the tubing and 5 N NaOH is added at 10-15 minute

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* The beef heart extract was prepared by the method of Dole (9) but was used without dialysis. It was stored in the cold under toluene.

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intervals to maintain the pH at about 7.5-8.0. Acid production ceases abruptly when the glucose is completely utilized.

The formation of acid and the production of streptokinase for a typical run are illustrated in Figure 1.

Separation and purification. When acid production by the culture has ceased the broth is either stored in the cold room over night or immediately treated as follows:

The culture is first centrifuged for 20 minutes to remove a white gummy mass which includes a large proportion of the cells. The supernatant is filtered with suction through a layer of Hy-Flo supercel about one centimeter in thickness. With care a sparkling clear filtrate can be obtained which contains all of the streptokinase activity of the whole culture.

This filtrate is adjusted to pH 4 with concentrated HCl. A cloudy precipitate forms immediately which is very difficult to remove. If, however, this suspension is placed in visking casing and dialyzed with rocking against cold, running tap water for two to three hours the cloudy precipitate flocculates and settles rapidly. The contents of the dialysis sacs are transferred to a tall storage bottle and allowed to settle. As much of the clear supernatant as is practicable is siphoned off and discarded and the precipitate is collected by centrifugation. The supernatant contains little or no activity.

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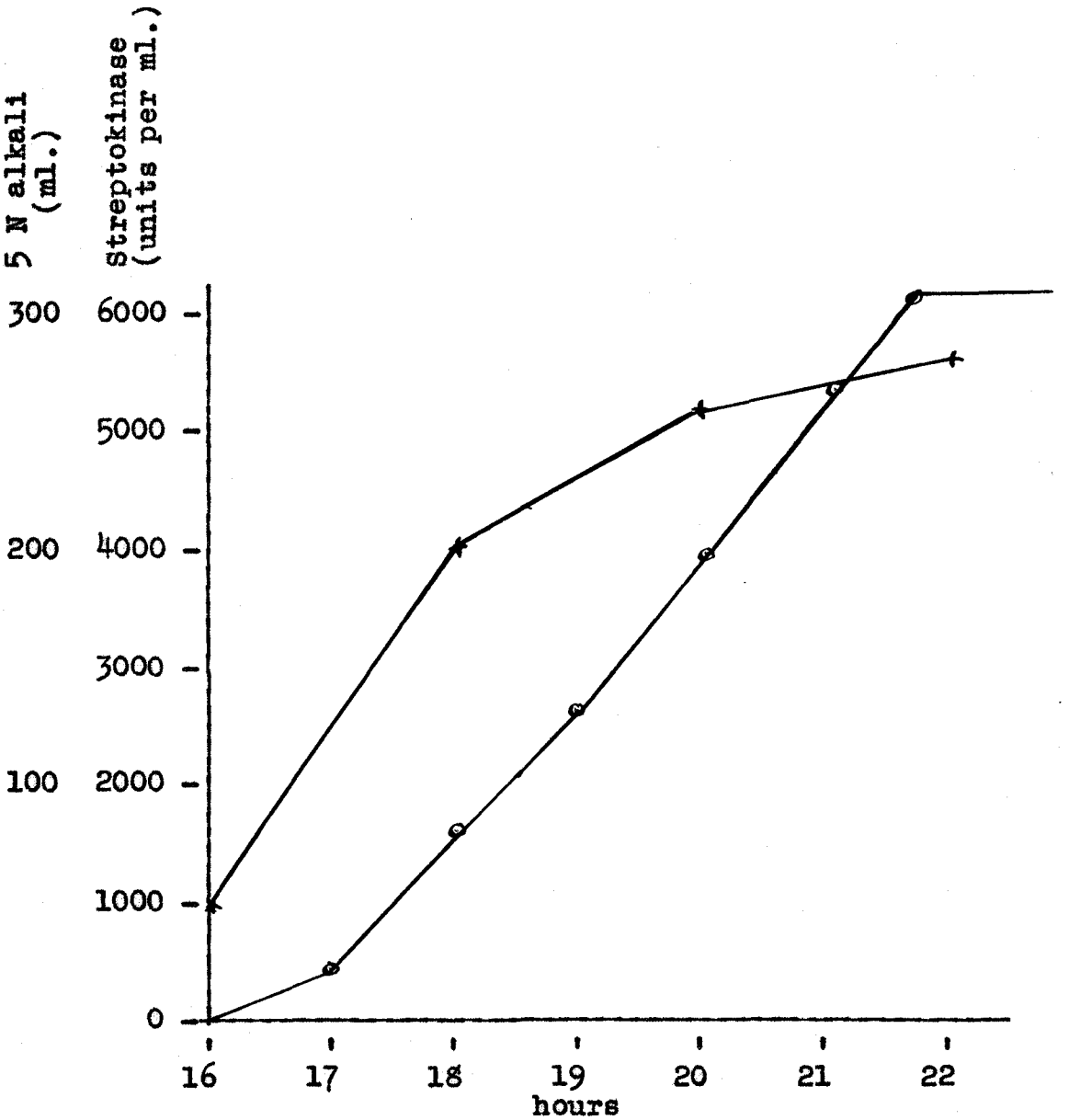

Fig. 1. Production of streptokinase by <u>streptococcus hemolyticus</u> H46A ✦ ; addition of alkali to culture ● .

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The greyish precipitate is then extracted with 0.1 M phosphate buffer at pH 7.6 and is filtered clear through supercel. The filtrate thus obtained contains about 50% of the total streptokinase activity of the original culture. Much better recovery can be obtained by adjusting the pH of the extraction mixture to about 9 for a brief period and then back to 7.6, but the resulting preparation is not as clean. (Dialysis and lyophilization at this stage yields a preparation having a potency of 1-2 units of streptokinase per µg. of solids.)

The clarified extract is brought to half saturation by the addition with stirring of an equal volume of saturated $(NH_4)_2SO_4$. The pH is adjusted to 5.6 with acetic acid and the mixture is allowed to stand at room temperature until flocculation is complete. The precipitate is collected by centrifugation and redissolved in buffer solution. The solution after thorough dialysis against running tap water and over night against distilled water is quick frozen and lyophilized. A pure white product having a streptokinase activity of about 6 units per µg. of solids or 60 units per µg. of nitrogen was obtained.

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Plasminogen preparations

Fibrinogen, crude. The fibrinogen used in the clot lysis studies (See - Assay of streptokinase under Methods) must necessarily be a crude preparation. It must also, of course, be free from active plasmin that would spontaneously lyse the clot.

Citrated whole human blood was used. The plasma was obtained by centrifugation and was treated with 1/4 volume of saturated $(HN_4)_2SO_4$. The precipitate was taken up in 0.1 M phosphate buffer and lyophilized. After two or three attempts a preparation was obtained which gave a clot that remained firm for at least 48 hours. This single preparation was used throughout this study.

Plasminogen. Plasminogen, as was noted earlier, is associated with the euglobulin fraction of the plasma proteins. It can thus be precipitated at its isoelectric point, pH 5.2, after reduction of the salt content either by dilution with distilled water or dialysis or it can be precipitated by 1/3 saturation with $(NH_4)_2SO_4$.

The product obtained by either of these techniques gives evidence of contamination with considerable amounts of protease inhibitor. Thus any study of the rate of activation of this product with streptokinase would be confused by the fact that the curve obtained is the resultant of

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the two reactions:

$$\text{Plasminogen} \xrightarrow{\text{streptokinase}} \text{plasmin}$$

$$\text{Plasmin} + \text{inhibitor} \longrightarrow \text{inactive complex}$$

Heating the plasminogen preparation at 56° C. destroys much of the inhibitor along with a considerable portion of the plasminogen itself (10).

Any attempt to purify the plasminogen would require large volumes of human plasma which were unfortunately not available. Consequently it was decided to use as starting material Harvard Fraction III of plasma proteins, which was reported by Christensen (11) to contain relatively concentrated plasminogen.

Fraction III* is largely insoluble in the saline phosphate buffer used in these experiments, but the active fraction is easily extracted by this solvent. It was thought originally that a simple extract might be satisfactory for use in rate of activation studies. However, it was found that concentrated extracts (10 mg./ml.) could not be activated by small amounts of streptokinase (5 μg./ml.), indicating possible presence of a streptokinase inhibitor.

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* Twenty grams of Fraction III was supplied by the Division of Biologic Laboratories, Department of Public Health, Commonwealth of Massachusetts through the courtesy of Dr. John M. Newell.

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With this assumption attempts were made to further purify the plasminogen in Fraction III extracts. A procedure used by Remmert and Cohen (12) for the preparation of plasminogen from serum yielded a satisfactory product.

Fraction III, 500 mg., was suspended in 10 ml. of 0.16 M NaCl at pH 7.2. The supernatant was decanted after centrifugation and the extraction repeated. The combined supernatants were diluted with an equal volume of distilled water to bring the salt concentration to 0.08 M. The pH was carefully adjusted to 5.2 with 1% acetic acid. The resulting precipitate was centrifuged off and the supernatant discarded. The precipitate was taken up in 5 ml. of whatever solvent was required for the particular experiment (i.e., saline phosphate buffer, borate buffer, or 0.16 M saline) and distributed in the required amounts, usually 0.5 ml., to stoppered test tubes. These tubes were stored in the frozen state at -30° C. The activity recovered, as measured in the presence of excess streptokinase, represented about 70% of that obtainable with the original Fraction III.

No diminution in activity was noted after two weeks in the deep freeze although the samples were used usually within a week.

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METHODS

Rate studies

In order to present comparable rate of reaction studies it is essential that the amount of product formed be related to the time of reaction as some straight line function. Christensen and MacLeod indicated that with some preparations of plasminogen, the activation by streptokinase exhibited the kinetics of a first order reaction. This was confirmed by Remmert and Cohen (12). Thus the logarithm of the concentration of the reactant, plasminogen, would be the required straight line function of time.

$\frac{dx}{dt} = k'(C_o-x)$ or by integration and transposition; $k' = 2.303 \frac{1}{t} \log \frac{C_o}{C_o-x}$.

C_o = total plasminogen

x = amount activated at time (t)

k' = specific rate constant*

The specific reaction rate constant is easily calculated from the slope of the plot of $\log \frac{C_o}{C_o-x}$ vs. t by multiplying by the factor 2.303. It was felt then that a comparison of the specific rate constant as affected by

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* k' is used rather than k to indicate the inclusion of the enzyme (streptokinase) concentration in the constant.

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different variables afforded the ideal method of studying the activation of plasminogen by streptokinase.

For the production of a curve that accurately describes the rate of activation of plasminogen by streptokinase the following conditions must be fulfilled.

1) The measurement of the concentration of the activated enzyme must be valid.

2) The activation must be capable of being stopped abruptly at a measured time and not allowed to continue during the measurement of the activated fraction.

3) The activated enzyme must be prevented from decomposition by autolysis or nonspecific denaturation.

These conditions will be discussed with regard to this problem in the order given.

The measurement of plasmin concentration. Unless an enzyme can be isolated and demonstrated to be a pure substance it is necessary to express its concentration in terms of its activity. Throughout these activation studies, the amount of proteolytic activity developed was measured by a modification of the Anson (13) method for trypsin. This method depends upon the formation of trichloroacetic acid soluble split products from urea-denatured hemoglobin. The concentration of these products is measured by the color developed with the Folin-Ciocalteau reagent (14).

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It was found that the presence of 40% urea used by Anson for the denaturation of hemoglobin greatly inhibited the activity of plasmin. Isoelectric casein, 1% solution in 0.1 M phosphate buffer at pH 7.4, gave about five times the amount of chromogenic products for the same quantity of enzyme. This was used as the substrate throughout this work.

The development of the color described by Anson leads to a rapidly fading blue color. The modification introduced by Heidelberger and McPherson (15) gave a much more stable color and was adopted as more convenient and reliable.

In summary then, the method used for the measurement of plasmin activity is as follows:

1) The plasmin is allowed to react in a 1% solution of casein for a given period. The reaction is stopped by the addition of an equal volume of 10% trichloroacetic acid. The tube is mixed by inversion and after 20-30 minutes is filtered through Schleicher and Schull #576 filter paper.

2) Three ml. of the clear filtrate is transferred to an Evelyn colorimetric tube. Nine ml. of 12.5% Na_2CO_3 is added and the color developed by the addition of 2 ml. of a 1 to 4 dilution of the Folin-Ciocalteau phenol reagent.

3) After 30 minutes the color is read against a suitable blank in the Evelyn colorimeter with a 660 filter.

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The blank is prepared by the identical procedure with the exception that the trichloroacetic acid is added immediately after mixing the plasmin and casein.

4) The colorimeter readings in per cent transmission (T) are converted to optical density (D) by the use of the Beer-Lambert law:

$$\text{Log } \frac{Io}{I} \alpha \text{ concentration}$$

With the blank, Io, set at 100 the formula is:

$2 - \log T = D \alpha$ concentration.

Most authors using similar techniques prefer to quote protease concentrations in terms of the amount of 'tyrosine' liberated using a tyrosine standard curve. In this work, because of the nonspecificity of the phenol reagent and since plasmin concentration is regarded simply as a function of streptokinase activity, the D values were used directly or for the calculation of rate constants.

If an enzymatic reaction can be shown to be of zero order, i.e., independent of the concentration of its substrate, the amount of product formed in a given time is directly proportional to the enzyme concentration. The enzyme under these conditions is said to be saturated with substrate during the entire course of the reaction so that variations in concentration due to substrate decomposition are without effect on the rate.

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The following experiments were designed to determine whether or not the procedure outlined above yielded figures reliably proportional to the concentrations of formed plasmin.

One volume of completely activated plasmin solution was mixed with two volumes of 1.5% casein at 37° C. An aliquot was removed immediately and added to an equal volume of 10% trichloroacetic acid to serve as a blank. Further aliquots were removed at intervals and treated in a like manner. The precipitated protein was filtered off and the color developed in the filtrate as outlined above. The optical density (D) obtained with the blank set at 100% transmission (D = 0) is plotted in Figure 2 against time of precipitation. The reaction is seen to be of zero order throughout its course.

As a further check, varying amounts of plasminogen were placed in different tubes. Buffer was added to bring all tubes to standard volume and all were activated with excess streptokinase. The final volume of the activated plasmin was one ml. Five ml. of 1.2% casein were added and the incubation (60 minutes), precipitation and color development were carried out in the usual manner. Each tube was read against its own blank prepared in identical manner but without the 60 minutes incubation. In Figure 3 the optical

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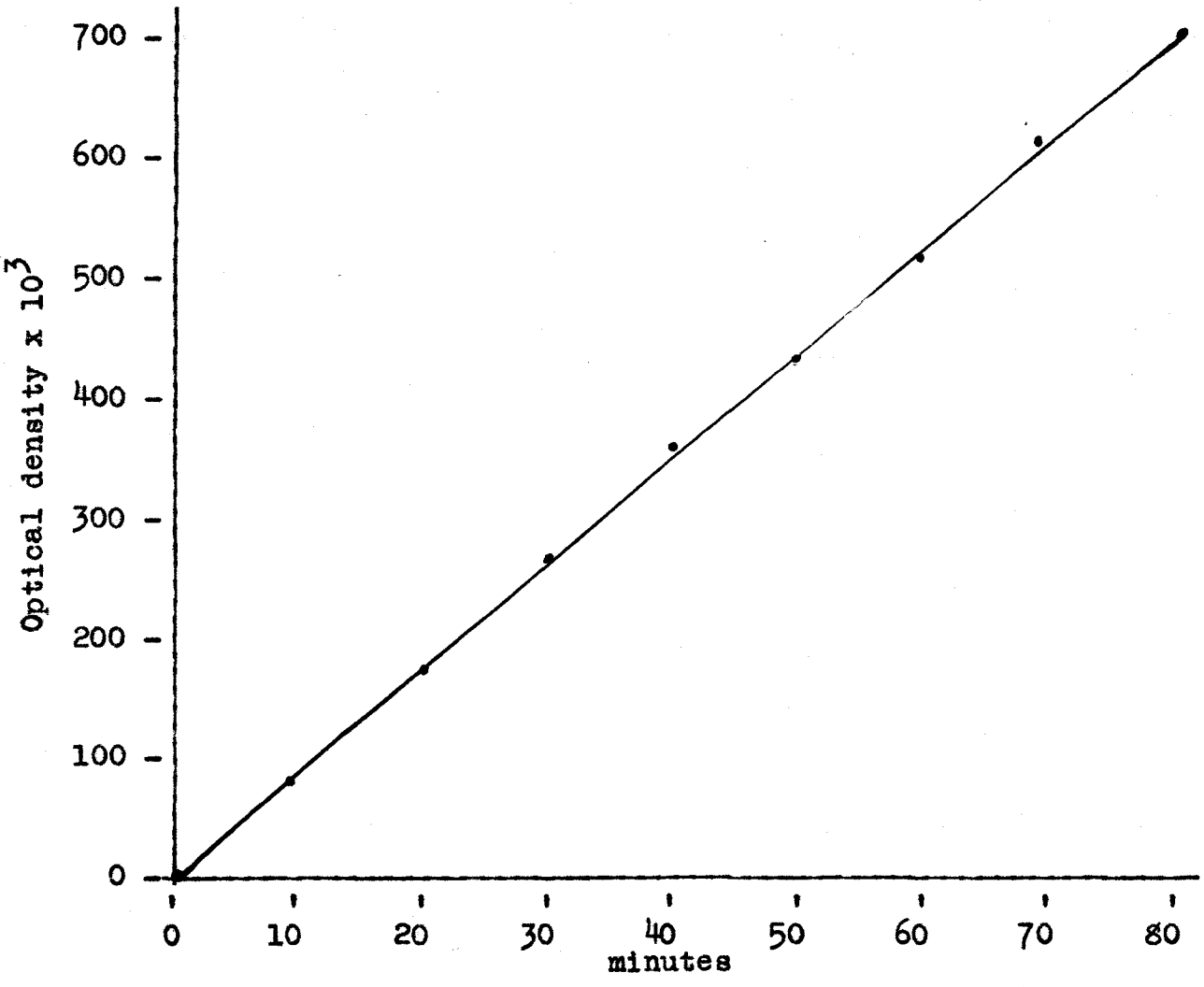

Fig. 2. Production of acid soluble chromogens from casein by plasmin.

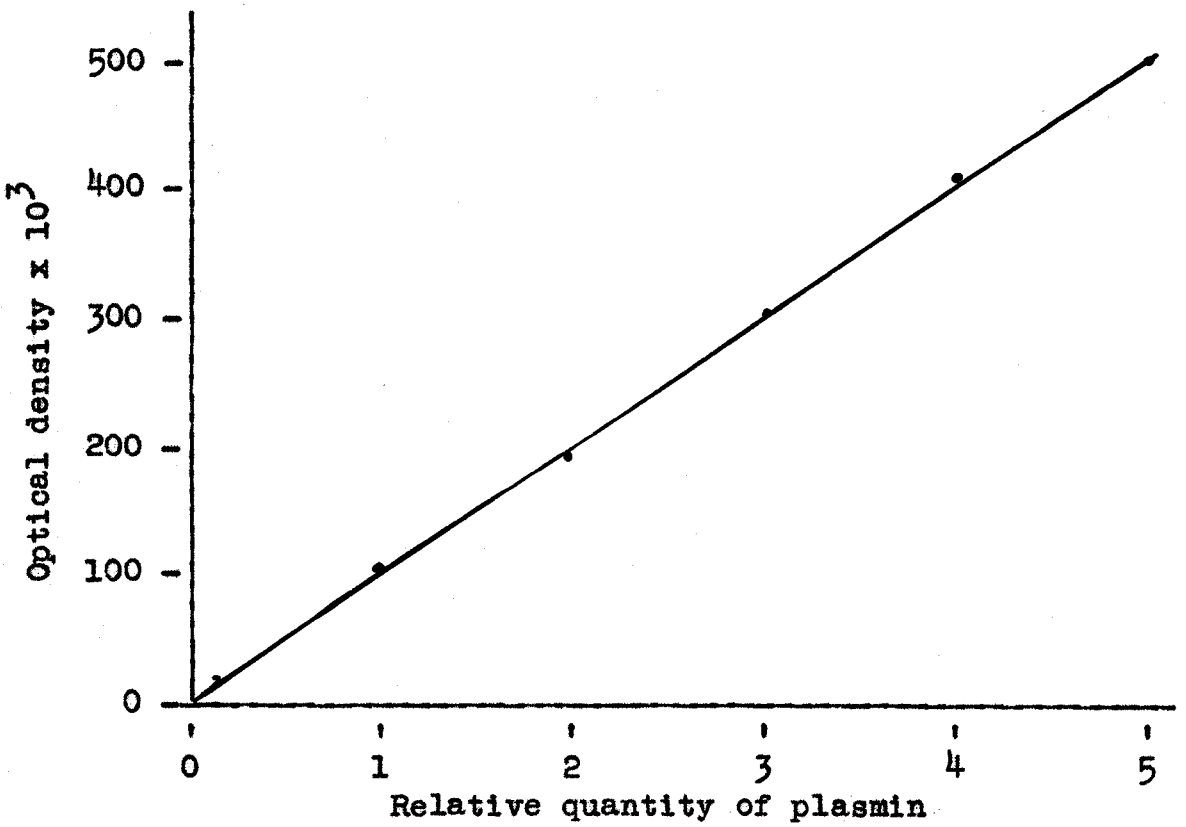

Fig. 3. Production of acid soluble chromogens by varying amounts of plasmin

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density is plotted against the relative concentration of the enzyme. It can be seen that the value is directly proportional to the concentration of the activated plasmin present.

Stopping the activation. The technique finally decided upon for terminating the activation of plasminogen by streptokinase was simply one of extensive dilution of the activation mixture with the substrate used for the measurement of the formed plasmin. The ratio of concentrations in the activation mixture to concentration in the digestion tube was one to thirty - that is 0.2 ml. activation mixture to 5.8 ml. of 1.035% casein.

A concentration of 5 μg./ml. of streptokinase gave a satisfactory activation rate whereas the 0.16 μg./ml. concentration in the presence of casein substrate gave a negligible activation.

Stability of plasmin. Purified plasmin after activation with streptokinase is quite unstable. Its solutions rapidly lose proteolytic activity on standing, particularly at 37° C. Whether this loss is due to autolysis or to denaturation was not determined. In any case such a reaction would seriously complicate rate studies.

During a study of the inhibition of plasmin activity by urea, mentioned earlier, it was observed that urea

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greatly retarded the inactivation of plasmin. A search for other substances showing this property led to the finding that glycine was even more effective. For example, it could be shown that an activated plasmin solution that lost 45% of its activity in one hour at 37° C. lost only 3.5% in the presence of 10% of glycine under the same conditions of temperature and pH.

Although glycine at this concentration seemed to inhibit the rate of activation somewhat, no other effect on the system was detected. The activation was clearly first order and the total activity obtainable with excess streptokinase was the same as without glycine.

Summary. The following procedure was used in all studies of the rate of activation of plasminogen by streptokinase. Where modifications, such as the addition of various reagents, were required the streptokinase was added in smaller volume of proportionately more concentrated solution.

One ml. of 20% glycine solution is added to 0.5 ml. of plasminogen in the proper buffer. 0.5 ml. of streptokinase at 20 µg./ml. is added and the contents of the tube are mixed by swirling. All reagents are added at the activation temperature. At timed intervals 0.2 ml. of the activation mixture is added to 5.8 ml. of 1.035% casein solution in 0.1 M phosphate buffer at pH 7.4. The casein-enzyme

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solution after 60 minutes incubation at 37° C. is treated as described under measurement of plasmin concentration, page 17.

The C_o value of the preparation or total plasminogen in 0.05 ml. is determined in the same manner except that streptokinase is added at a concentration of 1 mg./ml. Samples are withdrawn usually after 5, 10 and 15 minutes to insure that complete activation is obtained.

All colorimeter readings were made against an appropriate blank set at 100% transmission. The blanks were prepared by adding 0.2 ml. of the activation mixture to 5.8 ml. of casein and precipitating immediately with 6.0 ml. of 10% trichloroacetic acid.

The values obtained from the colorimeter in percent transmission are converted to optical density and these values are used directly in the plot of log $\frac{C_o}{C_o-x}$ vs. time.

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Assay of streptokinase activity

The method used for the assay of streptokinase followed that of Christensen (16) and other workers. It depends upon the fact that small amounts of plasminogen are present as contaminant in crude preparations of fibrinogen. Serial dilutions of streptokinase are added to a standard solution of fibrinogen and the fibrin clot is formed by the addition of thrombin. Streptokinase acting upon the plasminogen forms plasmin which causes liquefaction of the clot. The time required for complete liquefaction or lysis is inversely proportional to the amount of streptokinase present.

Procedure. One ml. of 0.2% fibrinogen (see preparations) in phosphate-saline buffer at pH 7.4 is measured into an 8 x 75 mm. precipitin tube, 0.2 ml. of the streptokinase dilution is added followed immediately by 0.1 ml. of 1% thrombin solution*. The mouth of the tube is covered by a small square of waxed paper and the contents are mixed by inversion. The tube is placed in a 37° C. water bath and is examined occasionally until the clot is formed. This usually takes 40-50 seconds. The clock is started at the formation of the clot and the time interval between formation and complete liquefaction of the clot is

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* Lyophilized bovine thrombin was supplied by the Lederle Laboratories of the American Cyanamid Co., Pearl River, N.Y.

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measured. This procedure is repeated with 4 or 5 serial dilutions of the streptokinase preparation.

The reciprocals of the dilutions used are plotted against their lysis times and the straight line connecting the points is drawn. The intersection of this line with the 30-minute abscissa is usually considered the end point. A line dropped from this intersection to the base line gives the dilution containing one 30-minute unit of streptokinase per 0.2 ml. Five times the reciprocal of this dilution gives the number of units per ml. of the original solution.

Sufficient repetitions for a statistical analysis of the error involved in this assay were not performed. However, it is estimated that in the hands of an experienced operator it is not greater than 5 or 10%.

Streptokinase units obtained by this technique cannot be considered referable to measurements made in other laboratories or made with different fibrinogen or thrombin preparations. They are useful only for comparison of preparations in various stages of purification or of streptokinase solutions treated with various reagents as in section 'Essential groups of streptokinase,' page 46.

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EXPERIMENTAL

Effect of hydrogen ion concentration on the rate of activation of plasminogen by streptokinase

The optimum pH for the activity of an enzyme as generally measured is the resultant of several factors:

1) The true optimum, or that pH at which the enzyme and its substrate interact most rapidly.

2) The effect of pH on the stability of the reactants or products.

3) Inhibiting effect of the accumulation of end products of the reaction.

Thus when the amount of end product formed in a given time is used as a measure of enzymatic activity, the pH optimum can vary widely depending upon the period of time chosen for the study. Obviously then the only valid measurement is of the rate of the enzymatic reaction in the initial stages, before the complications mentioned above become significant.

In these studies, it was found that the first order kinetics held quite well, at least in the initial stages of the reaction, throughout the range of pH used. From this it was assumed that the variation in the rate of enzyme-substrate interaction was the only significant function of pH during this phase.

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Materials. 1) Plasminogen was in an unbuffered solution 0.16 M with respect to NaCl and at a pH of approximately 7.2. It was prepared by the method outlined in the section on preparations, distributed 1 ml. per tube, and stored frozen.

2) Streptokinase was at a concentration of 40 μg./ml. in 0.004 M phosphate buffer at pH 7.4.

3) Other special reagents:

0.8 M Na_2HPO_4

0.8 M NaH_2PO_4

saturated NaOH

normal HCl

20% glycine in water adjusted to approximately pH 7.2 with NaOH.

Procedure. Two ml. of the glycine solution was added to the thawed plasminogen. The pH of the system was varied by the addition of 0.5 ml. of a mixture of the two phosphate solutions. Due to the tremendous buffering capacity of the glycine the addition of concentrated alkali to the phosphate mixture was required to attain the higher pH values.

Streptokinase solution, 0.5 ml., was then added and the timing was started. All reagents were added at 37° C. and this temperature was used throughout. At intervals of

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20 minutes, 0.2 ml. of the activation mixture was transferred to 5.8 ml. of casein substrate and the amount of formed plasmin determined as described under methods, page 17.

It was determined separately that the pH of the activation mixture did not change during the course of the incubation. Consequently the pH value obtained by means of the glass electrode, immediately after the last sample was drawn, was used. The ratios of the phosphates and alkali were varied arbitrarily as calculation of the resultant pH was difficult or impossible in such a complex system.

The effects of slight variations of pH during digestion of casein by the activated plasmin were nullified by the addition of small, experimentally determined amounts of normal HCl or alkali to the casein substrate. The pH at which plasmin concentration was measured was, consequently, always 7.4.

It was found that insignificant amounts of trichloroacetic acid soluble chromogenic products were formed during the activation period. Consequently a blank tube prepared at any point during the reaction was satisfactory for all tubes in the run.

The C_o value or total plasminogen was determined on the first and last days a given preparation was used. The procedure was similar to that of the other runs with the

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exception that the pH was always 7.6 and that a streptokinase solution containing 1 mg./ml. was used. Several samples were taken at about five minute intervals to insure that maximum activation was attained.

Results. The results obtained at several representative pH values are plotted as first order reactions in Figure 4. The specific reaction rate constant k' (min.) was calculated from the slope of these lines for the entire series of determinations and plotted against pH in Figure 5. The curve shows a rather sharp maximum at pH 9.

As can be seen from Figure 4 this is obviously not the optimum pH for the enzymatic formation of plasmin from plasminogen as the activity of plasmin falls off markedly at the higher pH values even in the presence of the stabilizing effect of glycine.

The actual significance of pH maxima determined in this way awaits further elucidation of protein structure and the determination of the centers of enzymatic activity of the protein molecule. At present it cannot even be determined whether the major pH effect is on the enzyme, its substrate, or on the enzyme-substrate complex.

However, the pH maximum of this system is definitely a property of the system, and as such may prove useful in differentiating between these preparations and plas-

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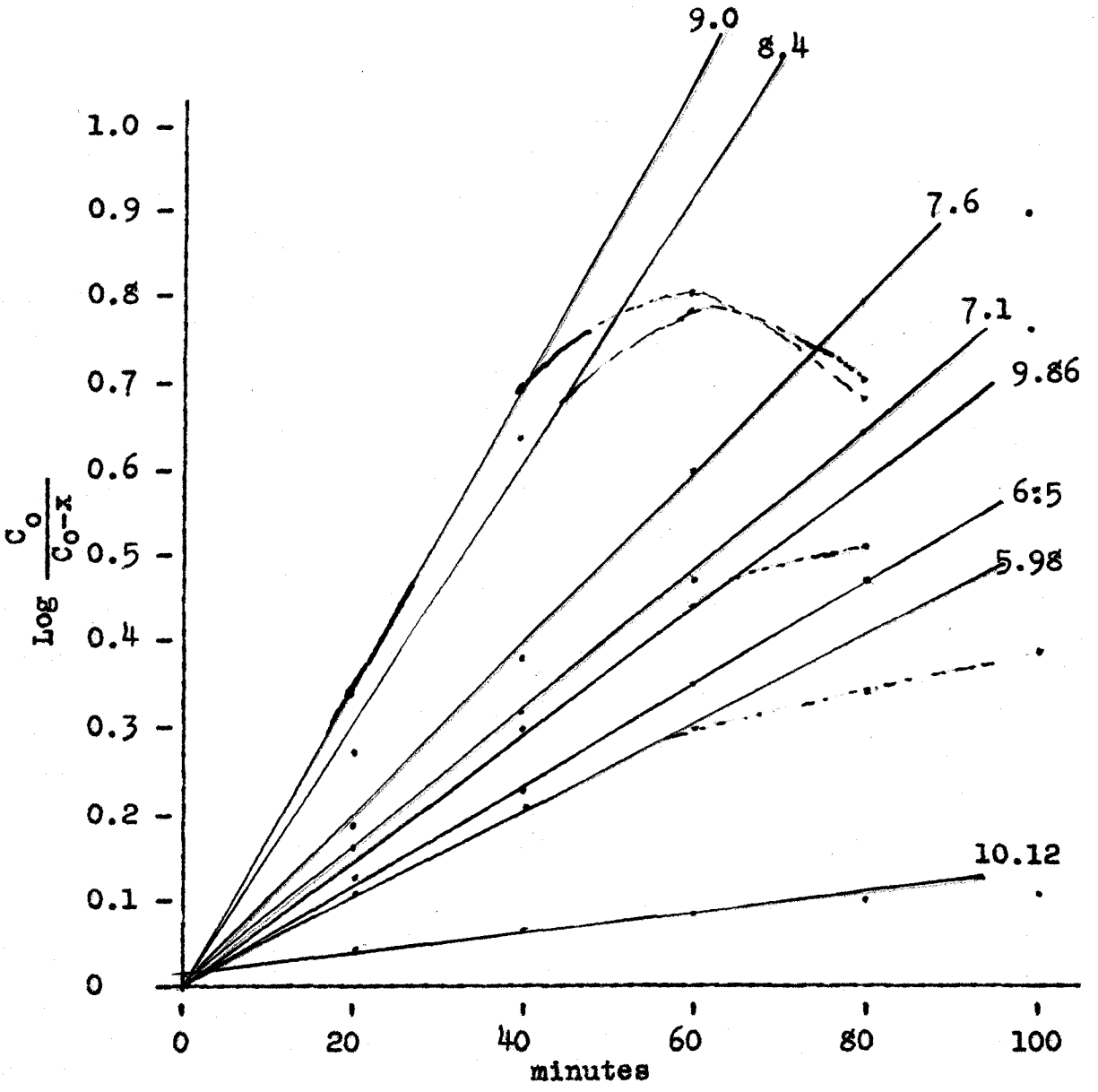

Fig. 4. Reaction rate curves for the activation of plasminogen by streptokinase at representative pH values.

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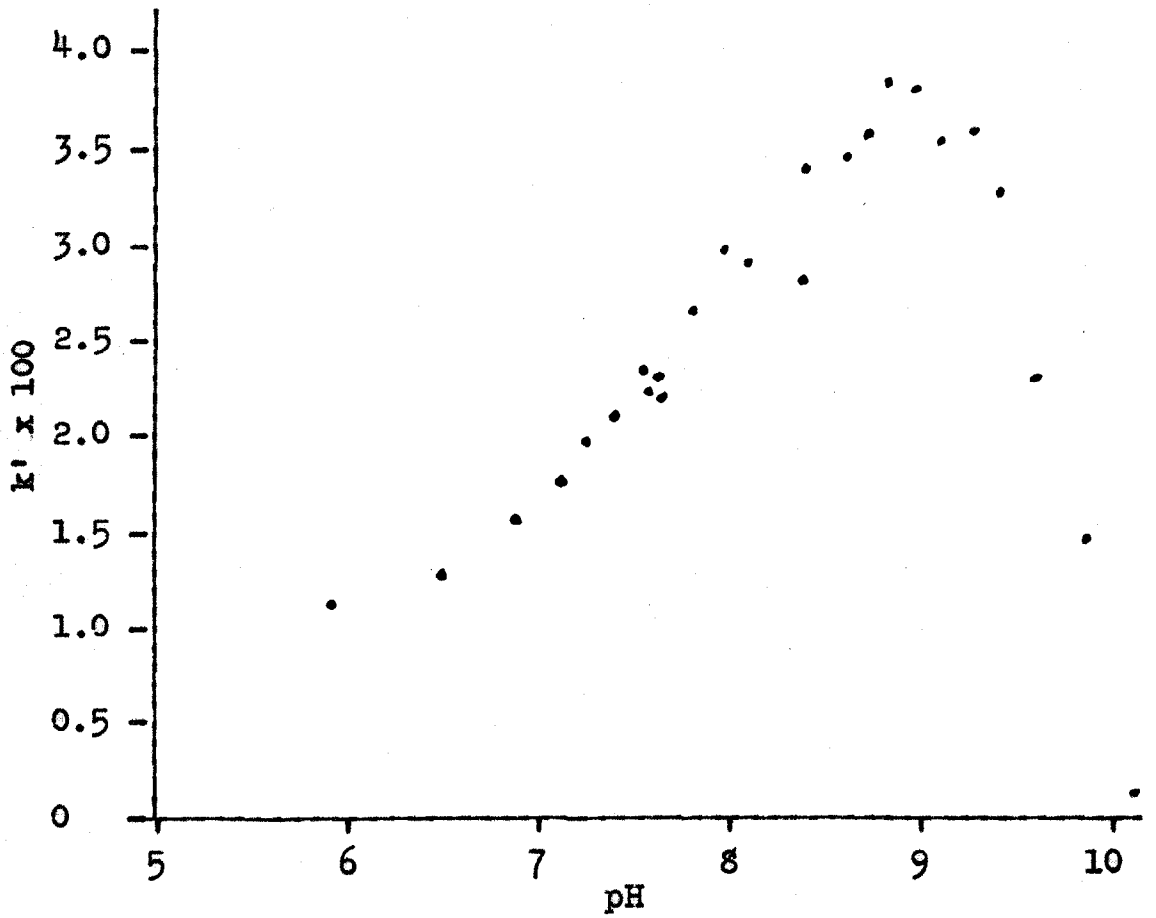

Fig. 5. Effect of pH on the specific reaction rate of the activation of plasminogen by streptokinase.

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minogens or kinases that may be obtained from different sources.

Effect of temperature

Much of the early work on the effect of temperature on an enzyme catalyzed reaction resulted in the assignment of an 'optimum temperature' for the reaction. Thus, if an enzyme and its substrate are allowed to react at various temperatures for a given length of time, a plot of temperature versus the amount of product formed will show a more or less sharp peak. This peak, however, varies inversely with the duration of the experiment being at the higher temperature for shorter incubations.

It was proposed as early as 1895 by Tammann (17) that two independent processes were simultaneously accelerated by temperature, i.e., the catalyzed reaction and the thermal inactivation of the enzyme. However, only recently has the concept of optimal temperature fallen into disuse. The present tendency is to study separately the heat inactivation of the enzyme and the heat effect on the catalyzed reaction.

Arrhenius in 1889 proposed the following equation as best fitting the available data on chemical kinetics as a function of temperature:

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$$\frac{d \ln k}{dT} = \frac{A}{RT^2}$$

k = reaction rate constant

T = absolute temperature

R = gas constant

A is a constant later designated as the activation energy and also referred to as $\triangle$Ha, E, or μ.

Integration of the Arrhenius equation and conversion to common logarithms gives the equation:

$$\log \frac{k}{k_1} = \frac{\mu}{2.3\ R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)$$

from which it is evident that the slope of the line obtained by plotting log k versus $\frac{1}{T}$, multiplied by 2.303 R or (4.58) is equal to the μ value or activation energy for the reaction in calories per mole.

In his excellent review of the effects of temperature on enzyme kinetics, Sizer (18) has demonstrated that enzyme catalyzed reactions follow the Arrhenius equation over a wide range of temperatures, up to the point where heat denaturation of the enzyme becomes apparent.

Another concept which is widely used by biologists is that of Q_{10} or the temperature coefficient of a reaction. This is simply the factor, usually between 1.3 and 3.5, by which the rate of reaction is increased by an increase of 10° in temperature.

Q_{10} and μ are of course related and one can be

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calculated from the other if the μ value for the reaction is known.

In the present work, where the concentration of the reaction product, plasmin, must be determined by its enzymic activity an additional complication, the thermal inactivation of plasmin, is involved. It was assumed that where, in the initial stages, the reaction satisfactorily followed the kinetics of a first order reaction the destruction of plasmin did not interfere significantly.

Materials. 1) Plasminogen prepared as described on page 12, taken up in phosphosaline buffer, and distributed 0.5 ml. per tube.

2) Streptokinase solution at a concentration of 20 μg./ml.

3) Glycine, 20% in phosphate buffer at pH 7.0.

Procedure. One ml. of the glycine solution was added to 0.5 ml. of plasminogen. This mixture was brought to the desired temperature and 0.5 ml. of streptokinase at the same temperature was added at zero time. At timed intervals 0.2 ml. of the activation mixture was added to 5.8 ml. of casein substrate at 37° C. and treated in the usual manner. The C_o value or total plasminogen was estimated as in the pH studies. The pH of all measurements in this series was 7.1.

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Temperature was controlled by means of a thermostat and blade heater with rapid stirring. Temperatures below 30° C. were maintained by immersing the entire water bath in a vat of ice water. The controls varied by about ± 0.5° C.

Results. The activation curves obtained are shown in Figure 6 and in Figure 7 the log of the rate constants obtained from these curves are plotted against the reciprocal of the absolute temperature. It can be seen that the activation follows the Arrhenius equation quite well over the temperature range considered.

Calculation of the energy of activation or μ value from the slope of the line in Figure 7 gives a value of 15,100 cal./mole. The Q_{10} between 30° and 40° is approximately 2.1.

Discussion. It is generally accepted that enzymatic reactions proceed by the formation of an enzyme substrate intermediate complex (19).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{reaction products.}$$

The question then arises as to which of these reactions, k_1 or k_3 is the pace setter and is characterized by the measured μ value.

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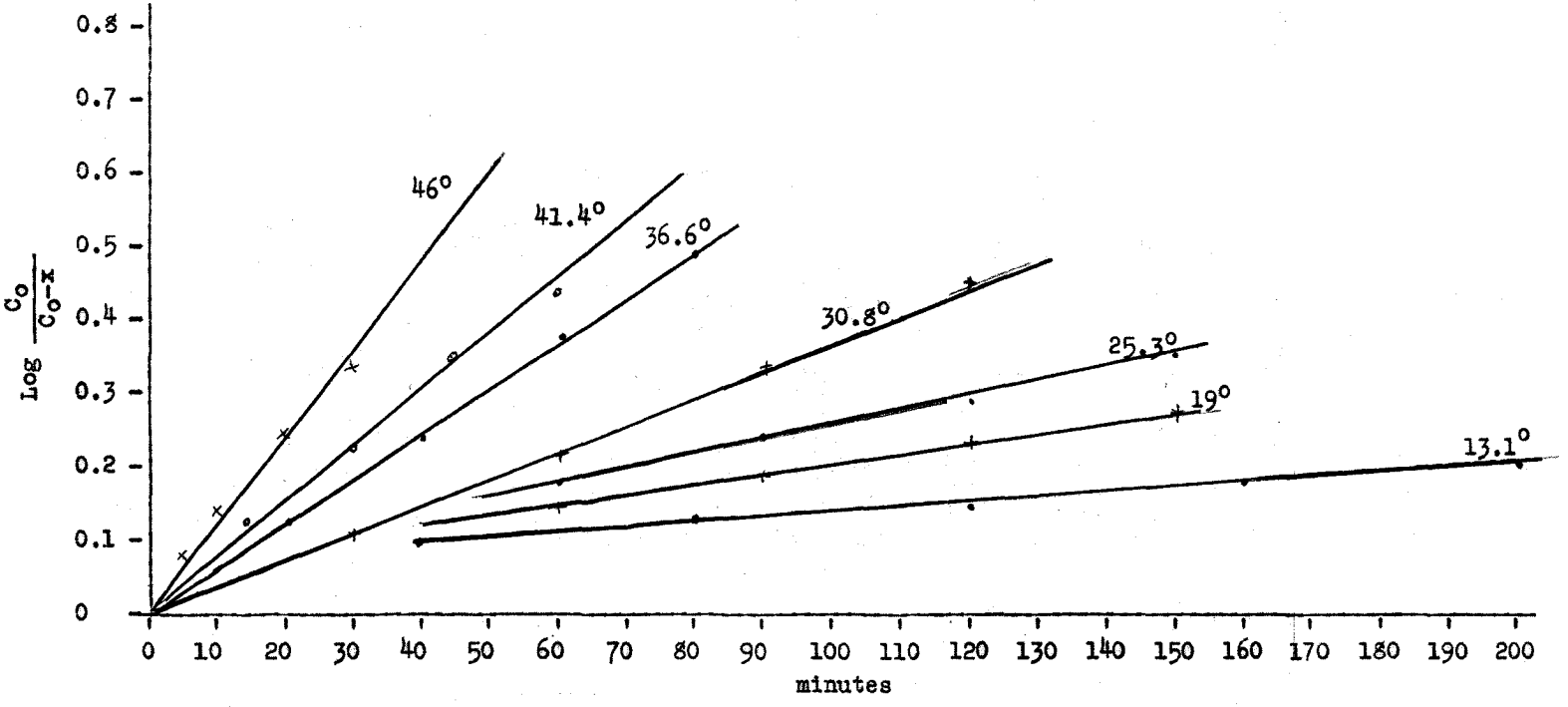

Fig. 6. Reaction rate curves for the activation of plasminogen by streptokinase at different temperatures.

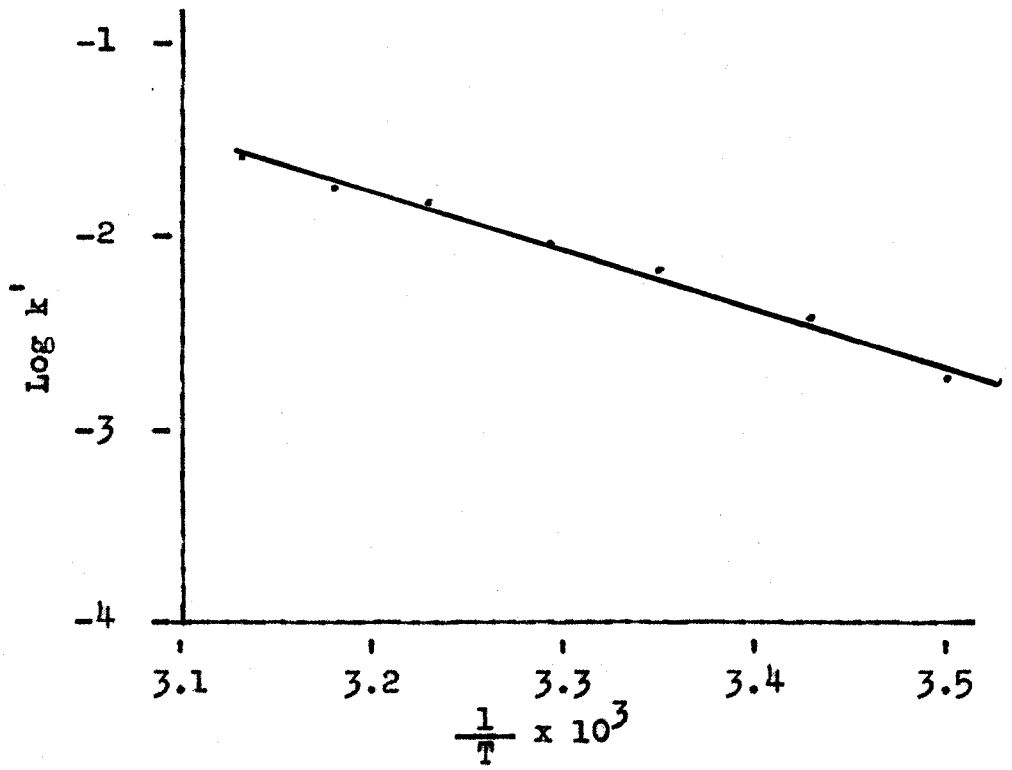

Fig. 7. Arrhenius plot for the activation of plasminogen by streptokinase.

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Certain tentative conclusions may be drawn from a consideration of the apparent order of the reaction. First order kinetics would be obtained if the reaction characterized by k_1 was considerably slower than that characterized by k_3 and was consequently the pace setter. In this case $(ES) \cong 0$ and its formation is by the amount of product formed.

$$\frac{d(ES)}{dt} = k_1(E)(S)$$

The μ value obtained would be a constant of the reaction:

$$E + S \longrightarrow ES$$

This problem has been extensively treated in reviews by Neurath and Schwert (20), Wilson (21), and by Bodansky (22).

The term μ is used in connection with biological reactions rather than E or A to indicate that there is no necessary implication of a physical meaning, e.g., energy of activation. Other terms used by biologists to avoid this connotation are critical increment of temperature, the temperature characteristic, or the temperature velocity constant.

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Effect of calcium and magnesium ions on the activation of plasminogen by streptokinase

A thorough survey of the effects of inorganic ions on the activation process was seriously hampered by the interference of certain ions in the determination of acid soluble chromogens by the Folin-Ciocalteau phenol reagent. Due to the weakness of the plasminogen preparations and to the obligatory presence of 10% glycine we were unsuccessful in adapting another method to the measurement of formed plasmin. Consequently this study is limited to those ions which gave no interference under the conditions used.

The presence of certain ions in the activation mixture is mandatory due to: 1) the buffer requirements, 2) insolubility of plasmin in distilled water, and 3) glycine stabilization effect. Thus in all solutions in these studies Na^+, K^+, borate, Cl^-, and glycine are present. It is the effect of the ion under consideration over and above any effects of these that was observed.

Materials.

1) Plasminogen in borate-KCl buffer at pH 7.4.

2) Streptokinase at 33 µg./ml.

3) 20% glycine in borate buffer

4) Molar Ca^{++} and Mg^{++} solutions adjusted experimentally to give a final value of pH 7.4 in the complete activation mixture.

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Procedure. To 0.5 ml. of plasminogen one ml. of glycine and 0.2 ml. of the ion solution were added. This was brought to a temperature of 37° C. and 0.3 ml. of streptokinase was added. Controls run simultaneously contained 0.2 ml. of borate buffer instead of the ion solution. At intervals 0.2 ml. of this mixture was transferred in the usual manner to 5.8 ml. casein substrate. In order to eliminate any effect of the ion on the casein digestion or on the development of color with the phenol reagent, an equivalent amount, 0.02 ml., of the ion solution was added to the casein substrate of the controls.

Results. The results obtained in the presence of 0.1 M Ca^{++} and Mg^{++} are shown in Figure 8 as compared with their controls. These cations were added as the acetate and sulfate respectively. The anions as their sodium salts were without effect. It can be seen that the rate of reaction is about doubled in the presence of either salt at 0.1 M concentration. The identity of this degree of stimulatory effect obtained with both ions is difficult to understand. It was likewise impossible to distinguish between the Ca^{++} and the Mg^{++} effect when both ions were used as their acetate salts.

Variation of the concentration of ion was studied with Ca^{++} only. The amount of activation in a given period

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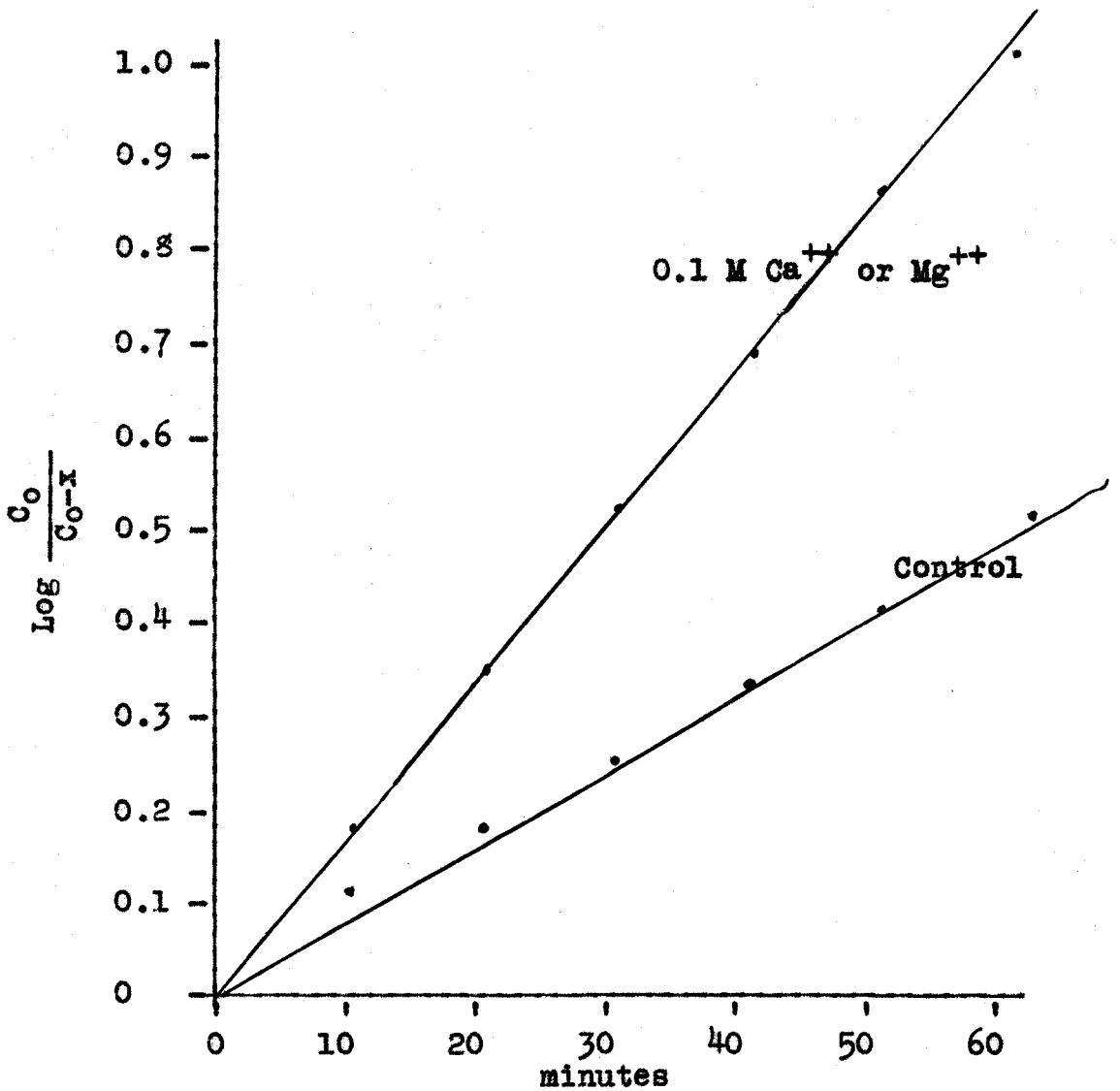

Fig. 8. Effect of 0.1 M Ca^{++} or Mg^{++} on specific reaction rate of the activation of plasminogen by streptokinase.

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was determined rather than a complete curve at each concentration.

The calculated amount of Ca^{++} solution was measured into the digest tube and the solvent removed by a stream of air in a boiling water bath. The dried tubes were transferred to a 37° C. water bath. A mixture of 0.5 ml. plasminogen, 1.0 ml. glycine, and 0.5 ml. streptokinase at 20 µg./ml. was prepared at 0° C. and 0.2 ml. was transferred to each of the tubes. After exactly 40 minutes, 5.8 ml. of casein substrate was added and the incubation continued for 60 minutes. Casein precipitation and color development were by the routine method. Figure 9 shows the effect of concentrations up to 0.1 M. Further work, not illustrated, showed that concentrations between 0.05 and 0.30 M were not significantly different in their effect on the activation rate.

Discussion. No interpretation of these results is possible at the present time. It will be recalled (23) that the autocatalytic activation of trypsinogen by trypsin is likewise stimulated by various ions notably Ca^{++} and Mg^{++}. However, it is doubtful if there is anything basically similar in the two cases. Autocatalytic activation of plasminogen by plasmin has not been observed although it is postulated in the slow activation of plasminogen following

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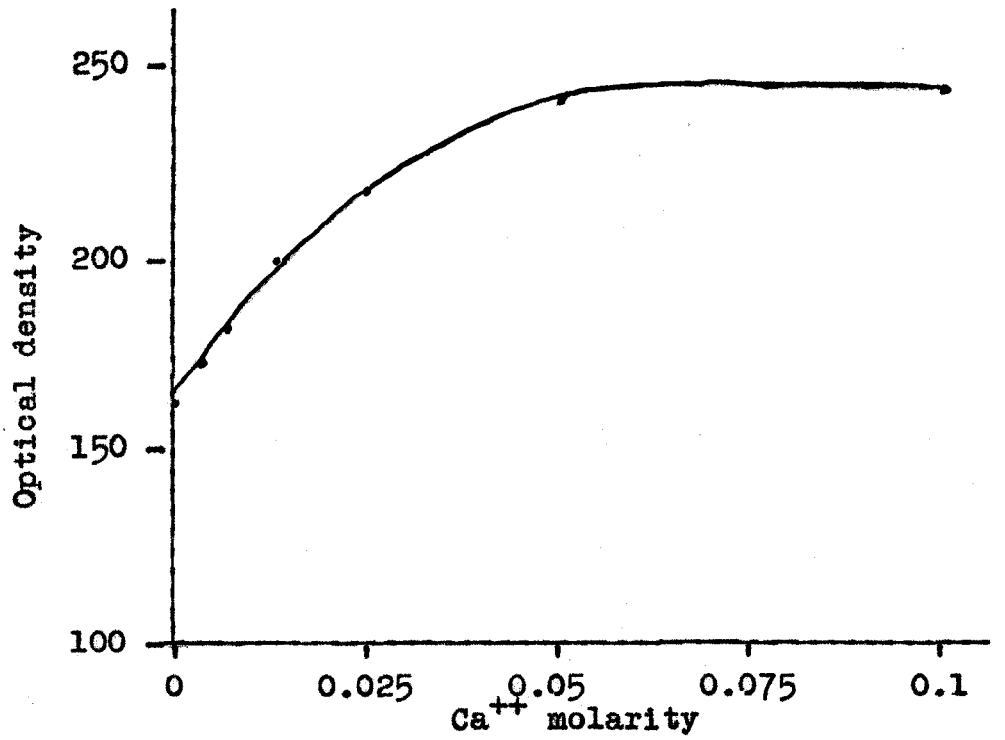

Fig. 9. Effect of varying Ca^{++} concentration on activation of plasminogen by streptokinase.

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chloroform treatment. Autocatalysis is certainly not an important factor in these studies.

MacDonald and Kunitz (24) were able to show that the presence of Ca^{++} ions greatly depressed the formation of 'inert protein' from trypsinogen. This resulted in an increased total trypsin activity. In these experiments the total plasmin activity (C_o) obtainable with excess streptokinase was not affected by the presence of Ca^{++} or Mg^{++}. The single observed effect was one of increased rate of activation of plasminogen by small amounts of streptokinase.

This work should be extended and amplified with the use of more highly purified plasminogen and a simpler activation system. In addition the effect of these ions on the spontaneous or chloroform initiated plasminogen activation should be studied.

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The essential groups of streptokinase

It is generally held today that proteins containing only the usual 'L' amino acids in peptide linkage may possess highly specific biological properties and that enzymatic activity does not necessarily depend, as originally believed, on the presence of a non-protein prosthetic group.

Assuming that the α-amino and the α-carboxyl groups of the various amino acids are largely tied up in the peptide linkage, the specific reactivity of a pure protein must be a property of the side chains. In the aliphatic mono-amino, mono-carboxylic amino acids the hydrocarbon chain is relatively inert. However, the more complicated amino acids possess side chain groupings of a more reactive nature. The effect of modification of one of these groups by a specific reagent on the activity of the protein determines the essential nature of that group.

Following this hypothesis investigators have attempted to determine what parts of the protein structure are essential for the biological activity. The problem resolves itself largely to the specificity of the various reagents used. Treatment resulting in hydrolysis of the peptide linkages or nonspecific denaturation of the protein of course gives us no information. On the other hand certain

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reagents under mild conditions cause such a striking loss of activity that many inferences can be drawn from their use.

In the present study a complication arises due to the fact that the enzymatic activity of streptokinase can only be manifested by the development of enzymatic activity in the substrate, plasminogen. Thus any reagent present might interfere either with the activity of streptokinase or with the activity of the formed plasmin. The observed results would be the same. Negative results, that is lack of inhibition, present no problem in this respect. Positive results, however, must be controlled by either removing the reagent after its action on streptokinase or by the construction of a time-inactivation curve in which little or no inhibition is evident in the early points.

The technique chosen for these studies was the streptokinase assay method described under methods. This technique not only gives quantitative results but also allows for large dilutions following treatment of the streptokinase which minimizes the effect of the reagent on plasmin.

At the present time there are, with the possible exception of some mild oxidizing or reducing agents, no reagents that can be considered absolutely specific for a

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given grouping. For this reason, only by the application of several reagents primarily affecting a given group can inferences be drawn. The experiments presented here are grouped according to what may be considered the primary action of the reagent employed.

Reagents primarily affecting free amino groups.

1) Formaldehyde. The reaction of formaldehyde with proteins is complicated (25) and its use as a specific reagent is therefore limited. The combination of formaldehyde with free amino groups in neutral solution is the basis of the 'formol titration' and it is generally held that this is the primary reaction. Evidence of possible interaction with guanidyl and sulfhydryl groups is summarized by Olcott and Franklel-Conrat (31).

Streptokinase solution at a concentration of 2 mg./ml. was prepared in 0.1 M phosphate buffer at pH 7.4. An aliquot was mixed with an equal volume of 0.2% formaldehyde and as a control an aliquot was mixed with an equal volume of distilled water. Both samples were incubated at 37° C. One ml. samples were withdrawn at the indicated times, diluted to 100 ml. and assayed immediately by the technique described under method, page 25. 0.5 ml. of 0.2% formaldehyde was added to the dilution mixture of the

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controls. Figure 10 shows the per cent of streptokinase activity retained versus time of incubation in 0.1% formaldehyde. The control showed no loss of activity in 180 minutes.

2) Nitrous acid. In addition to its oxidizing action nitrous acid reacts with free amino groups and free tyrosine (phenol) groups of proteins. According to Philpot and Small (26), in the presence of excess nitrite the reaction with amino groups is rapid and pseudo-bimolecular and that with phenol groups is slow and follows unimolecular kinetics. Weill and Caldwell (27) showed that at least with β amylase the oxidation by nitrous acid is reversible by sulfhydryl reagents or H_2S.

The technique used in these experiments was similar to that used by the above named authors. 23.1 mg. of streptokinase was dissolved in 2 ml. of 0.5 M Na acetate and 8 ml. of 0.5 M acetic acid was added. 5 ml. of this solution was added to 5 ml. of 2 M sodium nitrite and for the control 5 ml. was added to 5 ml. of water. The pH of the nitrous acid mixture was 4.2 and that of the control was 4.0. All reagents were mixed cold and the reaction carried out in the ice bath in the dark. At timed intervals 1 ml. of the reaction mixture was diluted to 50 ml. with

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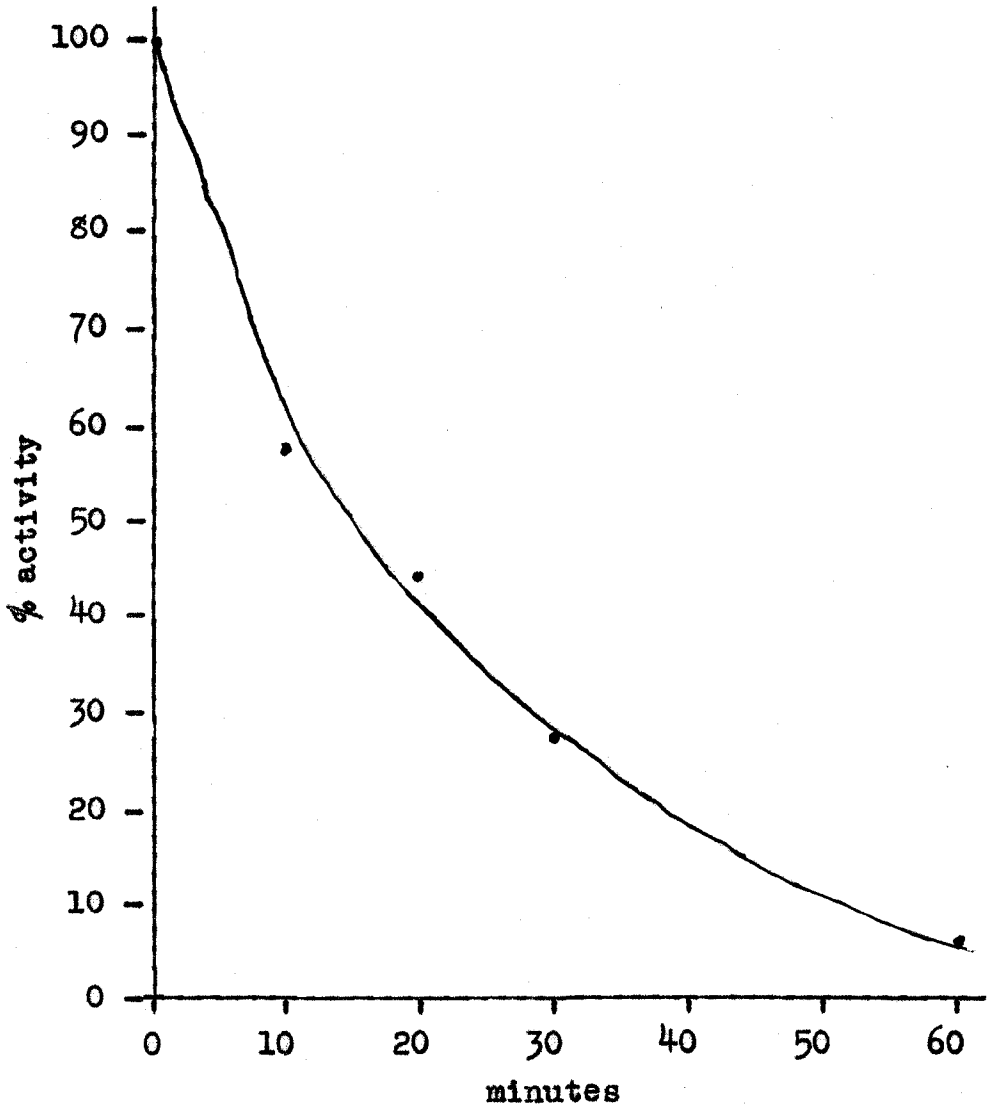

Fig. 10. Effect of 0.1% HCHO on streptokinase activity.

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0.1 M phosphate at pH 7.4 and assayed immediately for streptokinase activity. Figure 11a shows the per cent of original activity remaining at the times indicated. The plot of the reciprocal of the activity versus time, Figure 11b, shows fairly good agreement with the kinetics of a second order reaction.

Weill and Caldwell ascribe the irreversible inactivation of β amylase first detectable after 4 hours as due to the effect of nitrous acid on the phenol group of the tyrosine moiety. In these experiments the extreme rapidity of the reaction plus the second order kinetics suggest the involvement of free amino groups. Following experiments indicating the insensitivity of streptokinase to oxidizing or sulfhydryl reagents rule out the possibility of oxidative destruction by nitrous acid.

3) Phenyl isocyanate. Phenyl isocyanate reacts with the free amino groups of proteins in the following manner (28):

$$R\text{-}\underset{|}{C}H\text{-}NH_2 + OC{=}NC_6H_5 \longrightarrow R\text{-}\underset{|}{C}H\text{-}NHCONHC_6H_5$$

It has also been reported to react with sulfhydryl (29) and with phenolic groups (30) although the latter work has been disputed (31).

As in the experiments on nitrous acid, due to the absence or unessential nature of sulfhydryl groups in the

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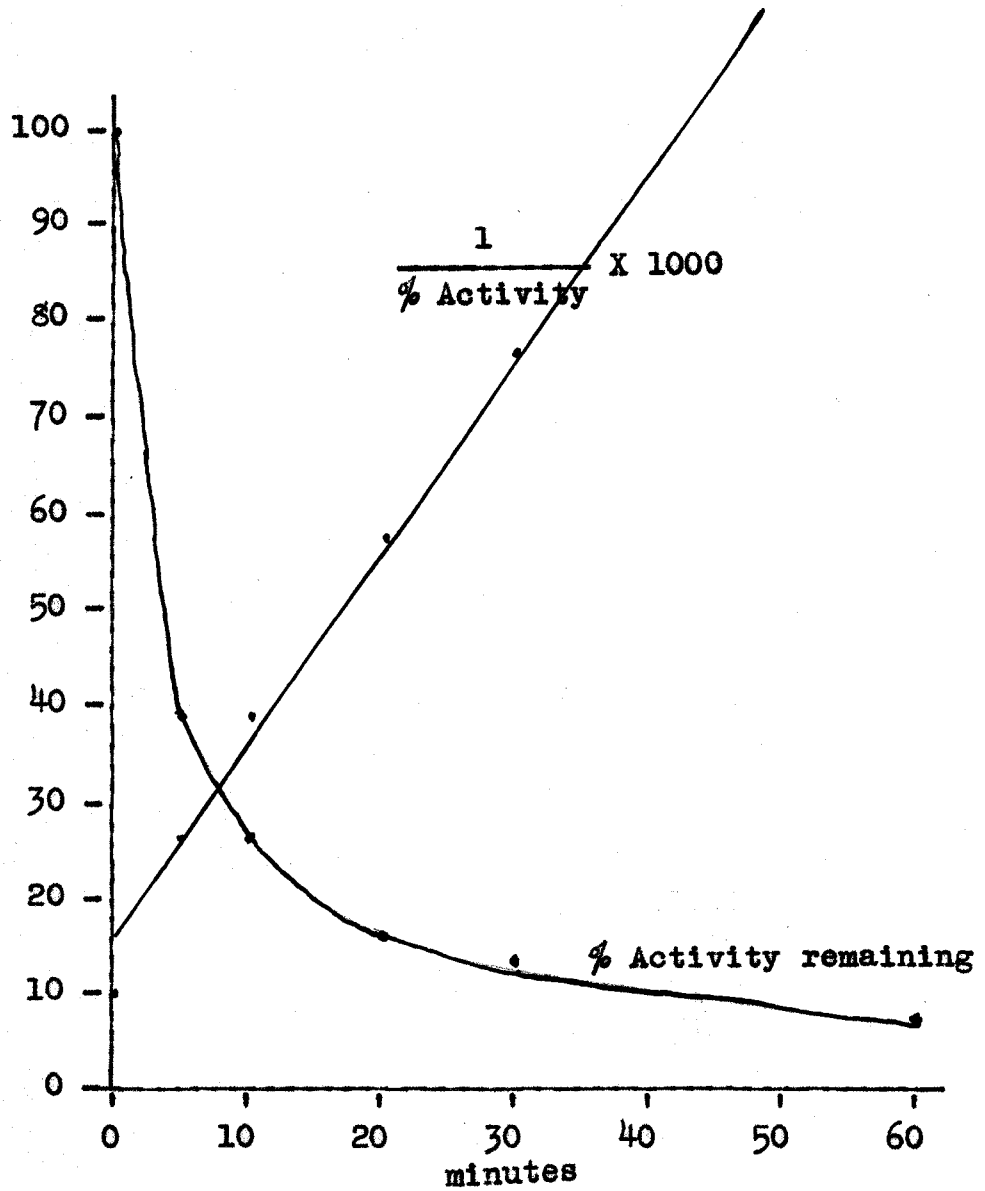

Fig. 11. Effect of nitrous acid on streptokinase activity.

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streptokinase molecule (See 'Reagents primarily affecting sulfhydryl groups'), any effect of phenyl isocyanate on streptokinase activity can be ascribed to its action on amino or possibly phenol groups.

The technique required for the use of phenyl isocyanate was derived from that used by Hopkins and Wormall (28) for the preparation of phenylureido casein. It does not lend itself to precise time-inactivation studies so that only data showing positive inhibition were obtained.

Five ml. of a solution containing 2 mg. streptokinase per ml. in 0.1 M phosphate buffer were placed in each of 3 tubes in the ice bath. Two drops of phenol red indicator were added to each. 0.1 ml. of phenyl isocyanate was added to tubes number 1 and 2. Number 3 was kept as a control. The contents of the tubes were mixed occasionally to keep the insoluble phenyl isocyanate in suspension as much as possible. Tenth normal NaOH was added as required to keep the pH between 7 and 8. After 60 and 120 minutes, tubes 1 and 2 respectively were centrifuged to remove the unreacted phenyl isocyanate and its reaction product with water, diphenyl urea. The contents of the tubes were transferred to visking casings and dialyzed 20 hours against running tap water. The control was carried along with tube

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number 2. After dialysis the contents of all the sacs were diluted to 100 ml. and tested for streptokinase activity.

The activity of the phenyl isocyanate treated streptokinase solutions was immeasurably low (less than 5 units per ml.) after dilution. The control was not appreciably affected, having about 600 units per ml.

Reagents primarily affecting sulfhydryl groups

Practically all protein reagents react with sulfhydryl (-SH) groups. The reagents discussed in this section are those for which some degree of specificity has been claimed. In these experiments, however, specificity is a matter of secondary importance. If a reagent known to attack sulfhydryl groups and possibly other groups of the protein molecule fails to inhibit the activity of an enzyme the conclusion can be drawn that SH groups are unessential to that enzyme. It is only when positive inhibition occurs that the problem of group specificity is involved.

1) Iodoacetic acid has been used extensively as a group reagent for proteins since Rapkine (32) first noted that it abolished the nitroprusside test for sulfhydryl groups. A thioether is formed by the reaction.

$PSH + ICH_2COO^- \quad PSCH_2COO^- + I^-$. The specificity of this reagent is discussed by Olcott and Fraenkel-Conrat (31).

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A streptokinase solution at 2.5 mg./ml. and pH 7.4 was treated at 25° C. for up to 60 minutes with 0.05 M iodoacetate. 0.4 ml. of the mixture was diluted to 100 ml. and tested for streptokinase activity. No loss was shown as a result of this treatment. A control, substituting water for the iodoacetate, likewise maintained full activity throughout the time of treatment.

2) p-Chloromercuribenzoic acid. Hellerman, Chinard, and Dietz (33) distinguished between the two types of sulfhydryl groups on the native urease molecule: the available groups or those free to react with nitroprusside, porphyrindin, or iodoacetate without loss of an enzymatic activity, and a second group less available to these reagents which was directly conconcerned with the urease activity. All SH groups were capable of combining with these reagents after guanidine denaturation of urease.

Those SH groups of the second or 'hidden' type reacted readily with p-chloromercuribenzoic acid with resultant loss of urease activity. A similar situation was shown by Singer and Barron (34) to exist with regard to the adenosine triphosphatase of myosin.

In order to determine whether streptokinase might by an enzyme of this type, p-chloromercuribenzoate was prepared and its activity toward streptokinase was tested.

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p-Chloromercuribenzoate was prepared by the oxidation of p-tolylmercuric chloride with alkaline potassium permanganate (35). The dried product was probably a mixture of the sodium salt and the free acid as it gave a sodium flame test and assayed 53.8% mercury as compared with 56.2% calculated for the free acid and 52.8% for the sodium salt. The product was made up in solution at 0.001 M with respect to mercury.

Streptokinase solutions at 2 mg./ml. and at a pH of 7.4 were treated with concentrations of p-chloromercuribenzoate up to 0.0005 M for 30 minutes at room temperature. After dilution of one to one hundred the mixtures were assayed for streptokinase activity. No loss in activity was noted. The activity of a preparation of the collagenase of Clostridium histolyticum was greatly inhibited by the p-chloromercuribenzoate solution used (36).

3) Ferricyanide and cupric ion. Weill and Caldwell (27) reported that potassium ferricyanide alone failed to inhibit β amylase, but when used in combination with catalytic amounts of cupric ion, it caused rapid, reversible loss of β amylase activity. This work confirmed the findings of Hellerman, Perkins, and Clark (37) for certain other enzymes which depend for their activities upon intact sulfhydryl

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groups.

Neither ferricyanide (1.66×10^{-4} molar) nor ferricyanide plus Cu^{++} (0.033 mg./ml.) had any measurable influence on the activity of streptokinase.

Iodination. Iodine has been used both as an oxidizing and as a substituting reagent. Its action on sulfhydryl groups is complicated and difficult to control. If one can eliminate the oxidation of sulfhydryl groups as a factor in the inactivation of streptokinase certain tentative conclusions can be drawn from the action of this reagent.

In neutral or alkaline solution iodine has found utility as a reagent for phenol groups (38). Its usefulness as a specific reagent for phenol groups is limited by the possibility of interaction with imidazole and indole groups. The many references to the use of iodine as a group reagent for proteins are summarized by Olcott and Fraenkel-Conrat (31).

Preliminary work indicated an extreme sensitivity of the clot lysis mechanism to iodine inactivation. Consequently the cumbersome step of dialyzing the reaction medium after iodine treatment of streptokinase was included. This complication precludes the possibility of accurately controlled treatment with iodine. The results obtained consequently can be considered only as a preliminary indication

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pending the development of a more precise technique.

Five ml. of streptokinase solution at a concentration of 2 mg./ml. in phosphate buffer at pH 7.4 was placed in each of four dialysis sacs. Standard iodine solution was added to each sac to give a final volume of 10 ml. and an iodine normality of: (1) 0, (2) 2.5×10^{-4}, (3) 2.5×10^{-5}, and (4) 2.5×10^{-6}. The sacs were tied off and dialyzed over night against running tap water. After dialysis the contents were diluted and tested for streptokinase activity by the usual method.

Sac #2 treated with iodine at an original concentration of 2.5×10^{-4} N retained not a trace of streptokinase activity. There was no loss of activity at the other concentrations. This sharp break was to be expected considering the wide (10-fold) differences in the original concentrations used. The clot lysis time of the control was not affected by dilution with material from sac #2 indicating the absence of free iodine.

Saturation of the inactivated streptokinase solution with H_2S did not restore any activity. Similar treatment of the control was without effect.

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SUMMARY

Methods for the preparation and purification of streptokinase and plasminogen are given. The activation of plasminogen by streptokinase is a first order reaction and the rate of activation is greatest at pH 9. The μ value or activation energy is 15,100 calories per mol for the reaction and the Q_{10} is 2.1. The rate of activation of plasminogen by streptokinase is greatly increased in the presence of Ca^{++} or Mg^{++} ions.

All rate studies were performed in the presence of 10% glycine which was necessary for the stabilization of active plasmin. Free amino groups and possibly the unsubstituted tyrosine group are necessary for the enzymatic activity of streptokinase. The presence of intact sulfhydryl groups is not essential.

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