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The proteolytic enzymes of proteus vulgaris.

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BOSTON UNIVERSITY

GRADUATE SCHOOL

Dissertation

THE PROTEOLYTIC ENZYMES OF PROTEUS VULGARIS

by

1412

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INTRODUCTION

There is, presently, a great deal of interest in the proteolytic enzymes produced by bacteria and their possible use in surgical debridement. Most of the investigations deal with the proteolytic enzymes of the anaerobes, such as the Clostridium group. It was thought that some attention should be given to the proteolytic enzymes of the aerobes. Strains of many aerobic bacteria were tested and several strains of Proteus vulgaris were chosen as being the best producers of the enzyme in question. Therefore, the purpose of this dissertation is to prepare filtrates of cultures of Proteus vulgaris rich in proteolytic enzymes, to isolate, purify, and study the properties of the enzymes.

HISTORICAL BACKGROUND

Production of Enzyme by Bacteria

Kendall and Walker (22) were the first investigators to study the nutritional requirements of P. vulgaris for the production of the proteolytic enzyme. They found that if dextrose was added to a gelatin medium, the enzyme was not produced until the dextrose had disappeared. The addition of a culture filtrate containing the enzyme to a dextrose-gelatin substrate had the same effect as on a gelatin substrate. They concluded that dextrose inhibits the formation but not the action of the enzyme. The authors then went on to state that the enzyme was liberated into the medium to hydrolyse proteins. The products then entered the cell to be used as a source of energy. Thus, when a sugar is introduced, it is used as the source of energy. Kendall et. al. (21) continued this work. They demonstrated by following the protein nitrogen, NPN, polypeptide nitrogen, amino nitrogen, and NH_3 in the growth medium and in gelatin hydrolysed by culture filtrates that the cell-free enzyme hydrolyses the gelatin without the formation of NH_3 . Jones (19) confirmed the fact that sugars inhibit enzyme production. He showed that the conclusion of Kendall et. al., as to the protein-sparing action of sugars, was justified since he found that sugars did not inhibit production of enzyme due to changes in pH as he maintained the same pH throughout the growth period.

Jones (20) later added to the proof by showing a decrease or complete lack of NH_3 or indol produced by the bacteria with increasing concentrations of sugars.

Haines (14) showed that NH_4^+ could be used as the only source of nitrogen in the production of the enzyme and was as effective as certain amino acids used singly.

Merrill and Clark (27) demonstrated that NH_4^+ could be used as the only source of nitrogen provided that Ca^{++} and Mg^{++} were present. Without these ions, the growth of bacteria was normal but no enzyme was found in the media. Haines (13) (14) (15) showed that Mg^{++} stimulated growth without increased enzyme production, while Ca^{++} increased enzyme production without an increase in growth. Gorini (11) studied this problem further. He found that the enzyme was inactivated by those anions which precipitate or form a complex with Ca^{++} . As the ionization constant or solubility product of the Ca - anion combination decreased, the amount of inactivation increased. If Ca^{++} is added immediately after the addition of the anion, there is no inactivation. However, if the Ca^{++} is added after some time has elapsed, there is no enzyme activity. He concluded that Ca^{++} served to stabilize and activate the enzyme. Citrate ion was not very effective in the inactivation of the enzyme of P. vulgaris X19.

There has been a difference of opinion as to the optimum pH for the production of the enzyme. Dernby (5) claimed that

it was pH 6.0 - 7.0, whereas Merrill and Clark (27) claimed it to be pH 8 - 9.

Properties of the Enzyme

Dernby (5) states that the optimum pH of enzyme activity is 6.5 - 7.5 for gelatin liquefaction and 7.0 - 7.5 for Witte peptone. Haines (14) (15) claims the activity to be greater at pH 8 than at 7.5 or 9 for gelatin hydrolysis. Gorini (11) claims the pH optimum to be 7 - 8 for gelatin.

Herter (16) was the first investigator to determine the effect of the enzyme on more than one substrate. He showed that the enzyme hydrolysed casein as well as gelatin but failed to hydrolyse egg albumin. Jones (19) found that gelatin, casein, and fibrin were hydrolysed and that the hydrolysis of all three ran parallel. He concluded that the hydrolysis was brought about by only one enzyme. Although the hemolysis of erythrocytes was included as a test of the proteinase, he could not be certain of the absence of a lipase.

Many bacteria, especially anaerobes, produce proteolytic enzymes which are activated by Fe^{++} , cysteine, and maximally by a combination of both. Weil et. al. (33) claimed that the proteinase of P. vulgaris was activated in the same manner.

Jones (19) found that there was some activity remaining after heating the enzyme at 100° C. for 5 minutes and that it was completely destroyed only after heating at 100° C. for 15 minutes.

Purification of the Enzyme

Only two investigators have attempted the purification of the enzyme. Jones (19) prepared a white powder which was active in dilutions of 1:10,000 by removing the water from a culture filtrate in a desiccator in the presence of H_2SO_4 , rubbing the powder with 95% ethyl alcohol to remove peptones, and then filtering and drying the resultant precipitate. Walker (32) precipitated the enzyme with safranin. The precipitate was active for 1 month when washed with absolute alcohol and dried. The author was unable to separate the enzyme from the safranin. Nevertheless, the enzyme was active when placed in protein solutions.

EXPERIMENTAL INVESTIGATION

I. THE DETERMINATION OF PROTEOLYTIC ACTIVITY

Many methods have been described in the literature for the determination of proteolytic activity. It was, therefore, necessary to make a critical survey in order to determine which methods would give the most information and yet be practical for the determination of a large number of samples.

It seemed necessary to select one method to use as a rapid screening process. This method, although not strictly quantitative, would have to show differences in enzyme activity. Those assays which require serial dilutions in order to determine the end-point were considered too cumbersome. That which was finally chosen was a modification of the procedure of Burdon (2).

A. Film-Strip Method

Reagents:

1. A developed and fixed X-ray film cut into 1" x 1/8" strips.

2. Buffer solution:

During the earliest part of this work, a phosphate buffer at pH 7.0 was used. Later, a 0.2 M tris (hydroxymethyl) aminomethane ("tris") buffer, made up to pH 7.4 according to Gomori (8), was used.

3. Sodium citrate: 10% solution.

Procedure:

Five ml. samples of dilutions of enzyme in buffer are placed in test tubes and 1 ml. of the citrate solution is added. The tubes are then placed in a water bath at 37° C. until the temperature of the test mixture has reached 37° C. The film strips are then placed in tubes at "zero" time. The time at which the film begins to clear is called "start" time. The time at which the film is completely clear is called the "finish" time.

1. Reproducibility of Method

It was first necessary to establish the reliability of this procedure. The enzyme used in this preliminary experiment was obtained by precipitating the enzyme from a 2-day culture of P. vulgaris by an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 5.5 and reconstituting the precipitate in 1/10 the original volume of phosphate buffer. Four tubes of the enzyme-buffer-citrate mixture were made up. In all four tubes the "start" time was 15 minutes and the "finish" time was 16 minutes.

2. Role of Sodium Citrate

The observation that sodium citrate reduced the time necessary to clear the film was, at first, thought to be due to an activating effect on the enzyme. This was shown not to be, since the citrate had no effect when the formol titration was used as a measure of activity. It was then thought that citrate acted as a complex-forming agent which removed some inhibiting cation. In order to test this

possibility, the effect of various cations was determined by adding 3 drops of a 5% solution of each of the appropriate salts to 1 ml. of a 5% sodium citrate solution, 4 ml. "tris," pH 7.4, and 3 ml. of enzyme. These solutions were then tested by the film-strip method. The results are given in Table I.

It can be seen that Ca^{++} , Ba^{++} , Mn^{++} , and Zn^{++} have no effect. Mg^{++} , Fe^{++} , Sn^{++} , and Cu^{++} cause some inactivation. It was concluded that none of the cations tested could explain the citrate "activation." The only remaining possibility was that enough of the colloidal silver from the film was oxidized to silver ions, which caused inactivation of the enzyme unless tied up in a complex with citrate. To show this, the next experiment was performed.

Five ml. of a dialysed culture filtrate were pipetted into each of three tubes. To the first was added 1 ml. of 10% sodium citrate; to the second, 1 ml. of a 10% NH_4Cl solution; and 1 ml. of H_2O , to the third. All solutions were adjusted to pH 7.4 and the film-strip method was then carried out. After 1 hour, the film was clear in the tube containing citrate. The film in the tube with NH_4^+ was clear after 3 hours, while the film in the tube in which only water was added did not clear after 6 hours of incubation. It has been observed that phosphate ions and amino acids were also effective in removing the Ag^+ inactivation, although not as effectively as the citrate ion.

TABLE I

The Effect of Cations on Enzyme Activity

Cation	None	Ca ⁺⁺	Ba ⁺⁺	Mn ⁺⁺	Zn ⁺⁺	Mg ⁺⁺	Fe ⁺⁺	Sn ⁺⁺	Cu ⁺⁺
time (mins.)	45	45	45	45	45	50	55	55	80

B. Formol Titration

There have been many good quantitative methods proposed for the measurement of proteolytic activity. However, most have one serious disadvantage and that is that only the trichloroacetic acid-soluble fraction is tested. Thus, any proteolytic activity occurring before reaching the stage of solubility in the trichloroacetic acid solution is not observed. Such assay methods include the biuret method (6), Moore and Stein's adaptation of the ninhydrin method (28), and Frame (7) and Russell's (30) sodium β -naphthaquinone procedure. Sherman and Neun (31), in an excellent article, have tested several of the better known methods and reported favorably on two of them: the Van Slyke amino nitrogen method and the Volhard-Löhlein procedure. This last method has the disadvantage mentioned previously. Pope (29) describes an assay using copper sulfate which, he claims, checks with the Van Slyke method. However, both the Van Slyke and the Pope methods are rather tedious to perform. For this study, the formol titration which also checks with the Van Slyke method (31) was chosen.

The problem of choosing a substrate then arose. Unfortunately, casein, bovine albumin and globulin, and many other easily obtainable and standardized proteins precipitate out at the pH obtained on the addition of formaldehyde. Bovine fibrinogen is not sufficiently soluble to be of any use, especially in kinetic studies. Gelatin, a substrate used

by a large majority of workers in the study of bacterial proteinases, was chosen as the most suitable protein substrate.

Two variations of the formol titration have been used for this work, both of which were adapted from the method of Iselin and Niemann (18). The first was performed by titrating to the phenolphthalein end-point using a semi-micro burette, while the second was a potentiometric titration using a micro burette.

Method I

Reagents:

1. "Tris" buffer, pH 7.4: The buffer is diluted 1:5 with distilled H₂O.
2. Gelatin substrate: A 3% solution of pure food gelatin is made in the dilute "tris" buffer. The solution is adjusted to pH 7.6, sterilized in the autoclave, and stored in a refrigerator until used.
3. Formaldehyde: adjust 35 - 40% formaldehyde to pH 7.0 using N NaOH.
4. Thymol crystals.
5. NaOH: Standard NaOH (approximately 0.01 N) is used to perform the titrations.
5. Phenolphthalein: A 0.2% solution in 95% ethyl alcohol.

Procedure:

To 4 ml. substrate add 1 ml. H_2O and 3 ml. enzyme solution. Add thymol by scratching the crystal with the thumb-nail in order to keep the particle size as small as possible. Mix by inverting the tube four to five times. Pipette 1.00 ml. of the mixture, in duplicate, into 2-dram vials, allowing equal drainage time for all samples. Add 1 ml. of formaldehyde and 1 drop only of the phenolphthalein solution. Titrate this mixture with 0.01 N NaOH until the first faint pink is observed. To perform the titration, use a semi-micro burette with a capillary tip, inserting the tip below the surface of the liquid to be titrated. The contents of the vial may be mixed during the titration by moving the burette with a circular motion. This motion causes the vial to spin. Wipe the burette tip after each titration.

The above constitutes the zero time titration. The remaining enzyme-substrate mixture is then incubated at 37° C. for four hours. At the end of this time, 1.00 ml. samples are removed and the titration is repeated. The difference in the number of microequivalents of NaOH used in the titrations is recorded.

1. Choice of Buffer

"Tris" buffer was chosen as the result of the following experiment: a 6% gelatin solution, pH 8.0, was made up to 3% by the addition of equal volumes of three different buffers: "tris," phosphate, and borate. The enzyme preparation was

obtained from $(\text{NH}_4)_2\text{SO}_4$ precipitation at pH 5.5 of a 3-day culture filtrate of P. vulgaris. The reconstituted precipitate was dialysed against running tap H_2O for 18 hours. Equal volumes of substrate and enzyme were mixed and allowed to incubate 18 hours at 37°C . The results will be found in Table II.

The fact that boiled enzyme did show activity will be explained later (pg. 46). A possible explanation for inhibition by phosphate can be found in papers that have been written on this subject. Gorini (11) demonstrated that Ca^{++} is necessary for the stability of the proteinase of P. vulgaris. This was confirmed by Merrill and Clark (27) and by Haines (13) (14) (15). Gorini showed that when substances which were known to precipitate calcium were added, there was a decrease in activity depending on how tightly bound the Ca^{++} became. Phosphates were included among those reagents which inhibit in this manner. However, there is no immediate explanation for the inhibition by the borate buffer.

2. Reproducibility of Method

A series of 10 tubes was prepared, all made up in the same manner. Each tube was titrated in duplicate. Calculations from the results give a standard error of the mean of ± 0.3 microeq.

3. Optimum pH for Enzyme Activity

The following experiment was performed in order to ascertain the pH optimum for the enzyme: A series of "tris" buffers

TABLE II

The Effect of Various Buffers on Enzyme Activity

Buffer	microeq. NaOH used
"Tris"	100
Phosphate	82
Borate	28
Boiled enzyme in Phosphate	22

was made. Aliquots of 6% gelatin were adjusted to a similar series of pH values. Equal volumes of "tris" buffer and gelatin having the same pH were then mixed and the pH was checked for each mixture. The enzyme used was derived from an alcoholic precipitation of a culture filtrate. The assay of activity was performed in the manner described.

The results, given in Table III, show that a pH of 7.4 is optimal for enzyme activity. It can be calculated that those values on either side of the optimum are just within the range of the standard error for the determination. Therefore, it is best stated that the optimal pH is in the range of 7.30 - 7.45.

The results in this experiment check well with the previous literature. Gorini (11) states that the optimum pH for activity is 7 - 8, whereas Dernby (5) claims it to be 6.5 - 7.5.

4. Correlation between Enzyme Concentration and microeq. NaOH Used

Various dilutions of a sample of enzyme were made. These samples were then assayed in the usual manner. The results are found in Graph I.

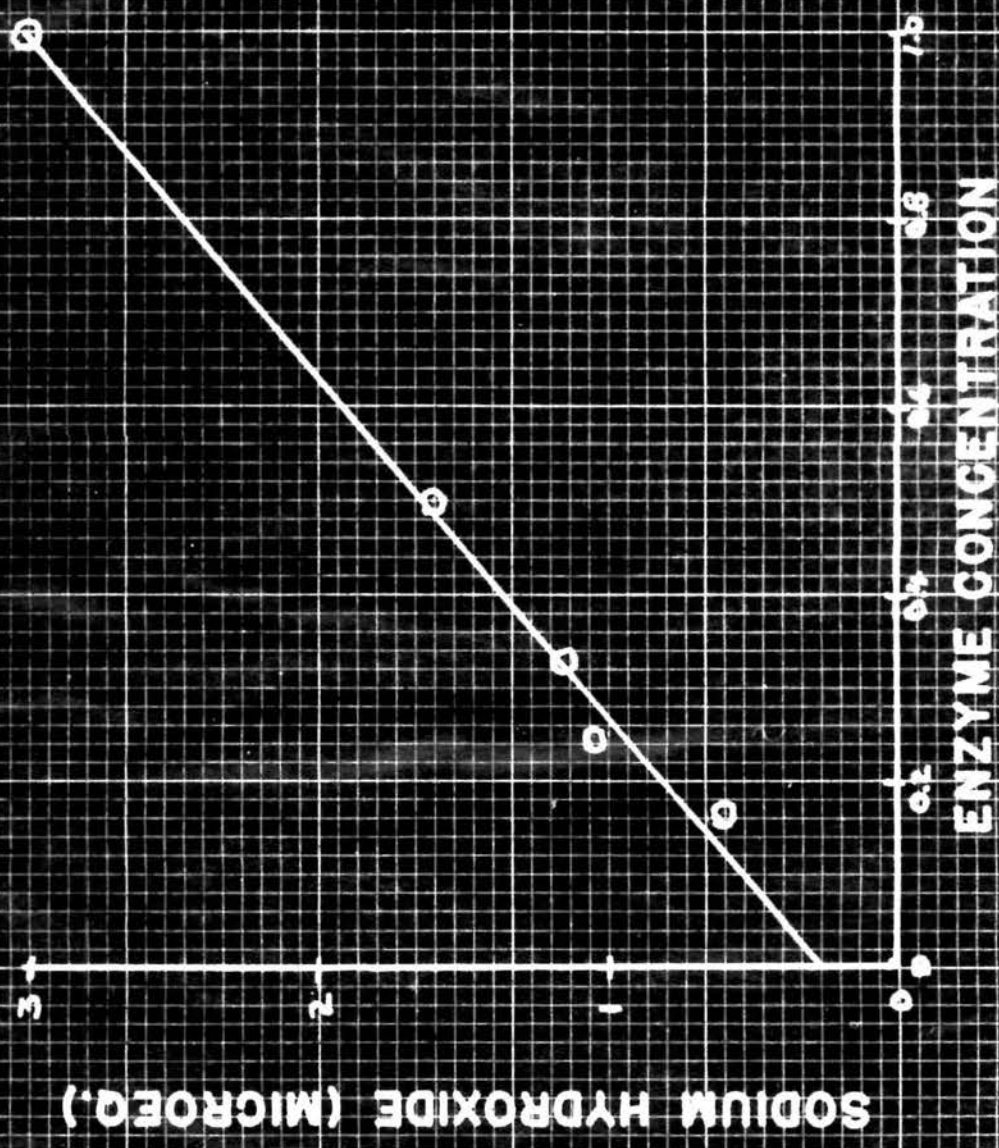
It can be seen from Graph I that there is a fairly good straight line relationship between enzyme concentration, in the range indicated, and assay value. The fact that the straight line does not go through zero is of no great consequence since the deviation is well within the error of the

TABLE III

The Effect of pH on Enzyme Activity

pH	microeq. NaOH used
8.0	1.5
7.80	1.7
7.70	1.4
7.45	2.2
7.40	2.6
7.30	2.2
7.22	1.6

Graph I. Correlation Between microeqs. of NaOH used and Enzyme Concentration for Method I.



determination. Furthermore, the lower values have little meaning, especially those below 1.0 microeq. NaOH.

C. Formol Titration - Method 2

Reagents:

1. Formaldehyde: 35 - 40% HCHO adjusted to pH 7.0 just before using or stored under liquid petrolatum.
2. Gelatin substrate: 60 gms. of Eastman Kodak purified gelatin is dissolved in 800 ml. hot water and cooled quickly. To this is added 25 ml. of 0.2 M "tris." The mixture is adjusted to pH 7.4 using 10% NaOH and the volume made up to 1000 ml. The substrate is placed in 100 ml. lots in 250 ml. flasks, sterilized in the autoclave and stored, sealed with Parafilm.
3. Thymol crystals.
4. NaOH: Standard 0.2 N NaOH stored under liquid petrolatum.
5. Caprylic alcohol.

Apparatus:

1. A Rehberg micro burette of 0.1 ml. capacity (Macalaster Bicknell Co., Boston).
2. A Beckman model H-2 pH meter equipped with 4990-29 and 4970-29 electrodes with special 24" leads.
3. Several Beckman 5 ml. beakers.
4. A 2" x 4" board with two rows of holes made to accommodate the beakers.
5. A tank of nitrogen.

The apparatus is set up as illustrated in Fig. 1. It is very important that the burette tip be below the surface of the liquid.

Method:

To a 3 ml. gelatin substrate are added 3 ml. H_2O , 2 ml. enzyme solution, and thymol, in the manner described previously. The contents of the tube are mixed by inverting several times. After 3 hours incubation at $37 \pm 0.1^\circ C.$, 1.00 ml. samples are removed and pipetted into the 5 ml. beakers which contain 1 ml. of the formaldehyde. A drop of caprylic alcohol is added to each beaker to prevent foaming. The samples are then titrated. The NaOH is slowly added while the beaker is manually rotated and a stream of N_2 is bubbled through the solution. When a pH of 7.80 is reached, the titration is complete. Subtract the titration value of a substrate-water control from the value obtained, to get the final value. The number of microliters thus obtained is then multiplied by the normality of the NaOH to obtain the results in microeq.

1. Choice of end point

A gelatin-water blank was made up and a 1.00 ml. sample plus 1 ml. formaldehyde was titrated. The plot in Graph II was obtained by recording the pH after the addition of various amounts of NaOH. The point of maximum slope, pH 7.80, was taken as the end point.

The Apparatus Used in the Second Method
of the Formol Titration

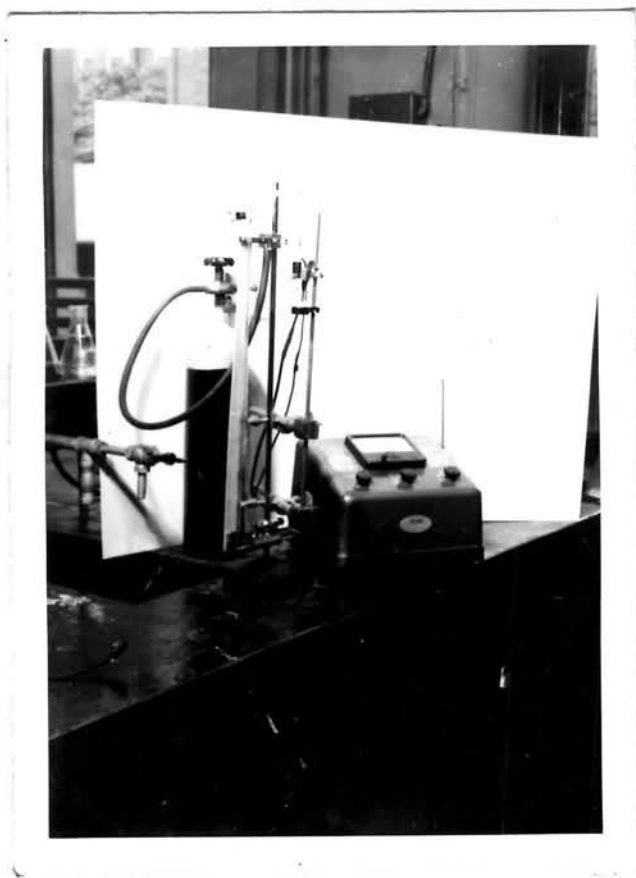
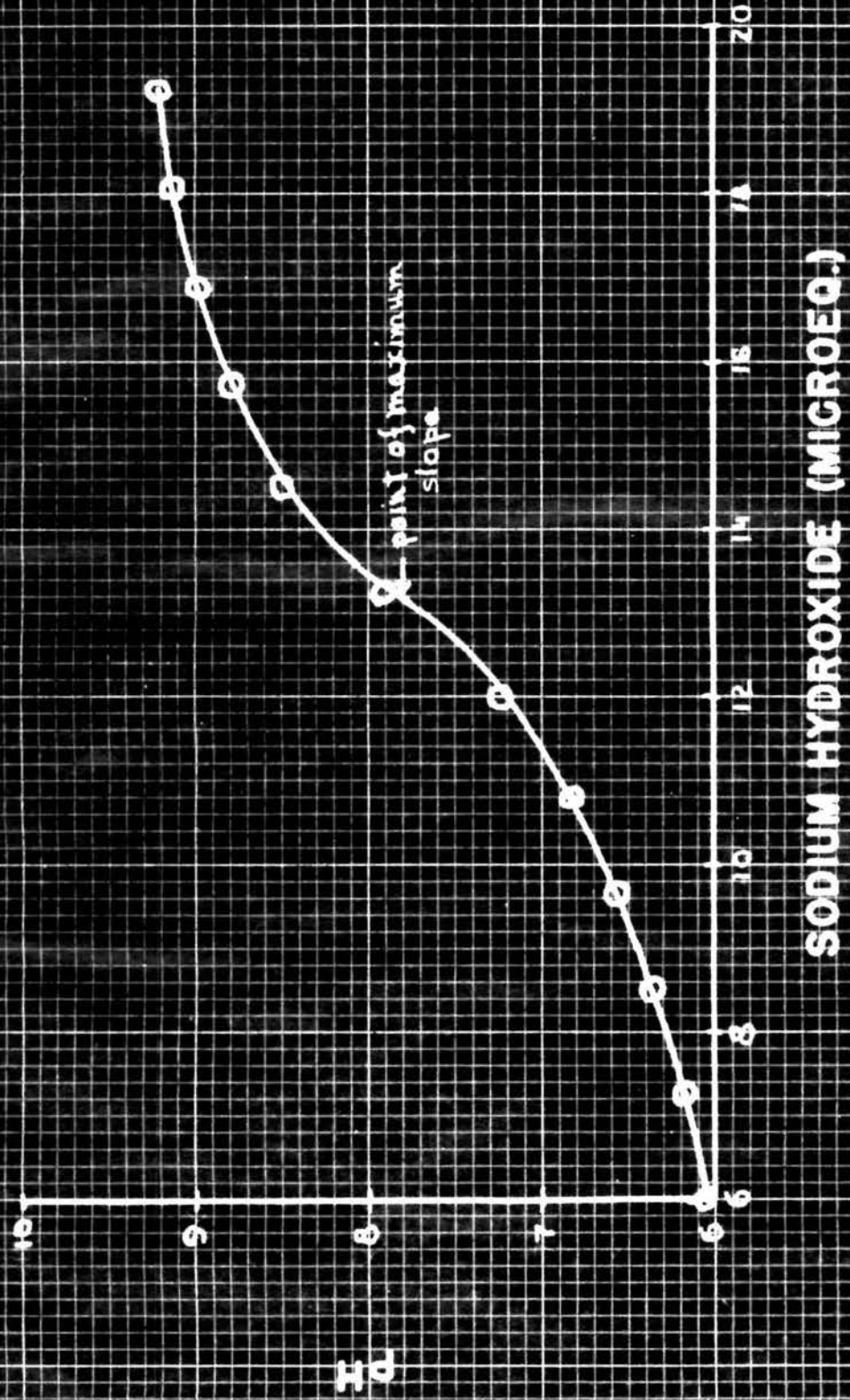


Fig. 1.

Graph II. Determination of End Point for Method 2.



2. Reproducibility of method

A test was made by performing six single determinations using identical preparations of enzyme for each. It was shown that even single determinations by this method are far superior to those obtained by the first method, since the standard error of the mean is ± 0.13 microeq.

3. Correlation between enzyme concentration and microeq. NaOH used

A series of varying concentration of enzyme was made and assayed in the prescribed manner. The results are found in Graph III.

It would seem from the plot in Graph III that, although the relationship between enzyme concentration and microeq. NaOH used is not a straight line, there is a good correlation at this relatively low enzyme concentration.

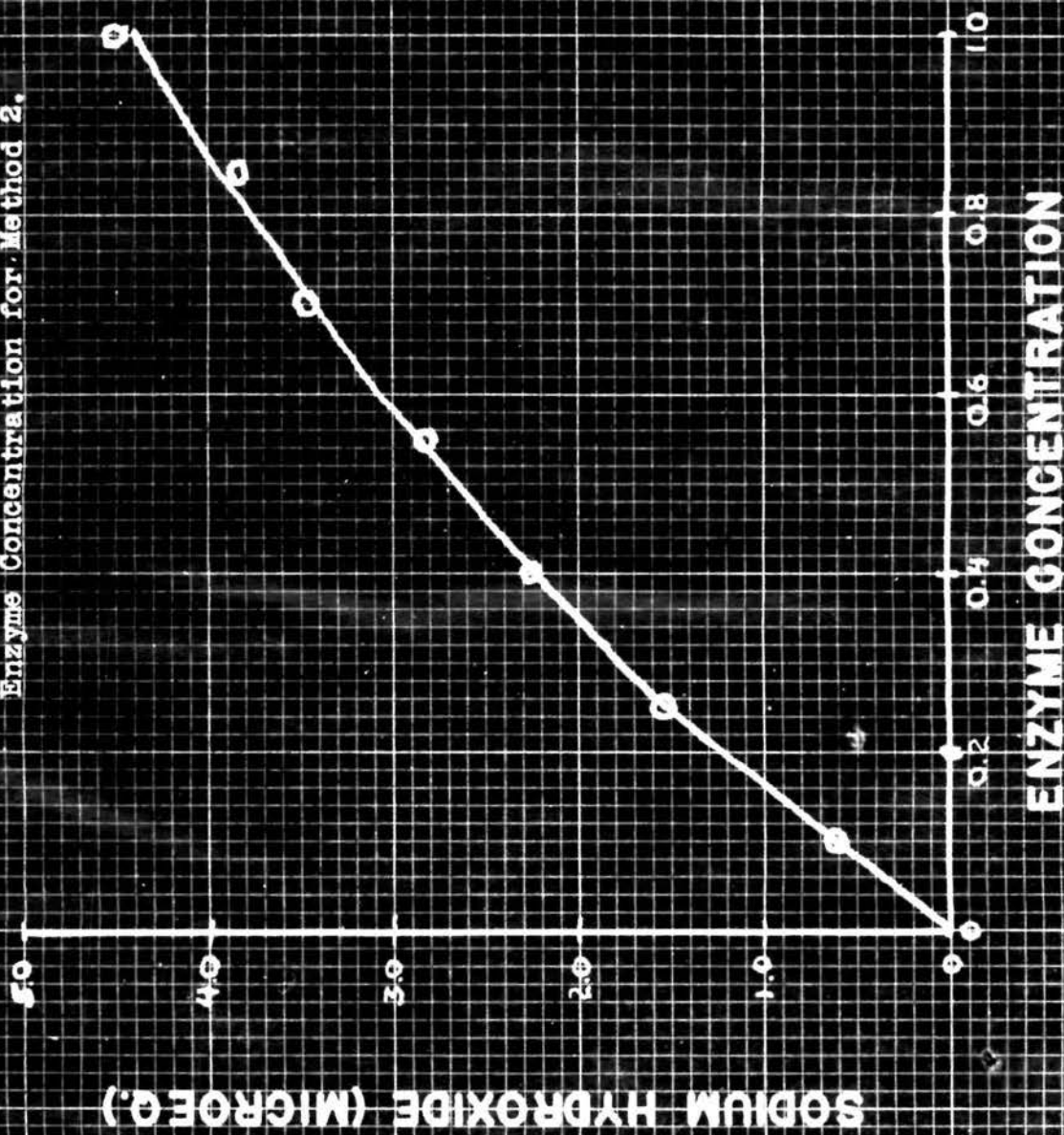
II. PURIFICATION OF THE PROTEOLYTIC ENZYME OF PROTEUS VULGARIS

A. Choice of Bacterial Filter

The first step in the purification of an enzyme from the medium in which bacteria are grown is the removal of the bacteria.

The relative value of three types of filters was tested, a Seitz filter 2" in diameter, a 4" Selas filter, and a 2" Mandler filter. As the culture was passed through the filter, the filtrate was collected in succeeding 17 ml. quantities. These were tested using the film-strip method.

Graph III. Correlation Between microeqs. of NaOH used and Enzyme Concentration for Method 2.



From the results listed in Table IV, it will be seen that the Mandler and Selas filters are equally effective in allowing the enzyme to pass through, while the Seitz filter tends to adsorb the enzyme.

B. Alcoholic Precipitation

The technique used in the addition of alcohol to the culture filtrate was a modification of that employed by Askonas (1). His apparatus was clumsy and allowed inadequate control of the rate of alcohol addition. The apparatus used in this work (see Fig. 2) consisted of a 50 ml. burette, the tip of which was connected to glass-tube coil. The other end of the coil projected above the surface of the ice-salt bath, in which the coil was immersed, and adjusted to deliver the precooled alcohol into the metal beaker. A stirring motor was used to agitate the ice water in order to prevent local over-heating around the beaker. A stirrer was also provided to prevent local high concentrations of alcohol in the beaker.

The burette was filled with 70% (v/v) ethyl alcohol or acetone and the stopcock opened until the coil was completely filled. The sample of dialysed culture filtrate was placed in the metal beaker and stirred slowly to prevent foaming. When the temperature of the culture filtrate fell below 1° C., the alcohol or acetone was run in, at a rate such that the temperature never exceeded 2° C., until the proper amount was added. The addition proceeded dropwise at first, but was added more rapidly after about 5 ml. had been added. (If

TABLE IV

The Effect of Bacterial Filters on the Filtration of Enzyme

Filter	Seitz		Mandler		Selas	
	ml.	ml.	ml.	ml.	ml.	ml.
start	>50	35 - 40	35 - 40	30	>50	30
finish		40	40	40		40

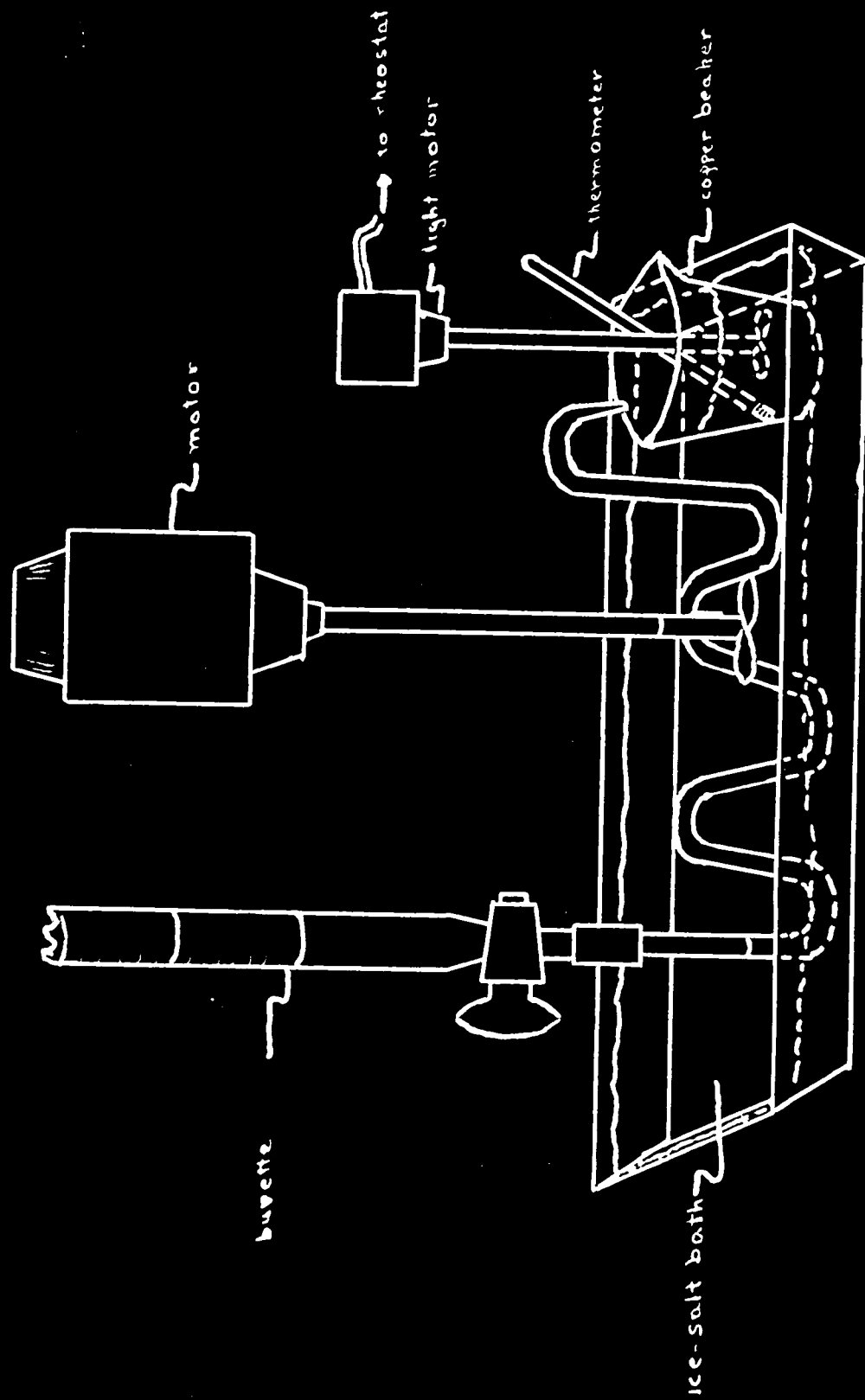


Fig. 2.

The Apparatus Used in the Precipitation
of Enzyme by Organic Solvents

two such set-ups are used simultaneously, it is possible to perform at least six precipitations per hour.) The suspension was centrifuged at 4000 R.P.M. for 10 minutes at 0° C. The precipitates were reconstituted to 1/10 the volume of the culture filtrate before dialysis.

It was found that the optimum conditions for precipitating the enzyme with alcohol were a 30% (v/v) final concentration of alcohol at pH 4.5 and a final ionic strength of 0.016. A 7-fold increase in purity was obtained with a 93.6% loss of activity. The optimum conditions for acetone were a 5% (v/v) final concentration of acetone at pH 5.0. The addition of a wide variety of cations did not improve the yield. A 9-fold increase in purity was thus obtained, but 93% of the enzyme was lost.

The above methods were abandoned in favor of the method described next.

D. Adsorption and Elution Using Kaolin

The use of kaolin in the purification of a proteolytic enzyme has been applied previously to the enzymes of the Clostridium group. Van Heynigen (17) adsorbed the enzyme of Cl. histolyticum at pH 5 and eluted it at pH 9 as the last step of his purification. Madinaveita (24) used kaolin to separate the spreading factor from the proteolytic enzyme of Cl. welchii. The proteinase was better adsorbed at pH 6 and eluted by 0.4% ammonia water. The spreading factor was best adsorbed at pH 4 and eluted by pyridine, without eluting the proteinase.

1. General technique

The general technique used in the isolation of the enzyme will now be described to avoid repetition later. Kaolin was added to a sample of dialysed culture filtrate, previously adjusted to the proper pH, and the mixture shaken for 20 mins. The mixture was then centrifuged and the supernatant liquid discarded. The kaolin was then washed by resuspending it in the proper solution, recentrifuging and then pouring off the supernatant liquid. The enzyme was eluted by resuspending the kaolin in the proper eluent and then removing the kaolin by centrifugation.

2. Optimum conditions for adsorption and washing

An experiment was performed to determine the optimum pH at which to adsorb the enzyme. This experiment consisted of adding 2 gms. of kaolin to 5, 90 ml. aliquots of the dialysed culture filtrate, previously adjusted to the pH values given in Table V by means of 10 ml. of McIlvaine citrate-phosphate buffer (26). The kaolin was washed twice with 30 ml. of a 1:3 dilution of McIlvaine buffer at the same pH used in the adsorption. The enzyme was eluted with 25 ml. of 0.3% ammonia water. The enzyme solution was filtered through Celite to remove the kaolin left in suspension after a preliminary centrifugation. This was done since the kaolin remaining in the ammonia water solution interfered with the nitrogen determination. The solution was dialysed overnight against running tap water.

TABLE V
The Effect of pH on Enzyme Adsorption

pH of adsorption	microeq. NaOH used	<u>mg. N</u> ml.	<u>microeq. NaOH</u> mg. N
5.0	4.8	0.0060	800
5.5	5.4	0.0058	930
6.0	3.8	0.0050	1100
6.5	1.8	0.0016	1120
7.0	0.6	0.0004	1500

The results in Table V show that the greatest amount of enzyme is obtained when pH 5.5 is used for adsorption. However, as the pH is increased, the purity increases. Despite this latter fact, pH 5.5 was chosen as the most valuable since a very much greater amount of enzyme is adsorbed at this pH.

The next experiment was performed by adsorbing the enzyme from 5, 90 ml. aliquots of dialysed culture filtrate, adjusted to pH 6.0 by 10 ml. of McIlvaine buffer. The kaolin was washed twice with 30 ml. of a 1:3 dilution of buffer at various pH values. The enzyme was eluted, filtered, and dialysed as before.

The results in Table VI show that as the pH of the wash solution is increased, the enzyme is eluted. Therefore, by increasing the pH of the wash solution, a purer enzyme preparation cannot be obtained without concurrent loss of enzyme.

3. Optimum amount of kaolin

To determine the proper amount of kaolin required to adsorb the enzyme, the following experiment was performed: Varying amounts of kaolin were added to 90 ml. aliquots of dialysed culture filtrate, adjusted to pH 5.5 with 10 ml. of McIlvaine buffer. The kaolin was washed twice with a 1:3 dilution of buffer at pH 5.5. The enzyme was eluted with 25 ml. of a 0.3% ammonia water solution. The eluent was dialysed overnight against running water. The results are found in Table VII.

TABLE VI
The Effect of pH of Wash

pH of wash	microeq. NaOH used	$\frac{\text{mg. N}}{\text{ml.}}$	$\frac{\text{microeq. NaOH}}{\text{mg. N}}$
6.0	3.8	0.0034	1100
6.5	2.2	0.0003	7300
7.0	0.8	0.0004	2000
7.5	0	0.0027	0
8.0	0	0	

TABLE VII
The Determination of Optimum Amount of Kaolin

kaolin (gms.)	microeq. NaOH used	$\frac{\text{mg. N}}{\text{ml.}}$	$\frac{\text{microeq. NaOH}}{\text{mg. N}}$
0.5	3.6	0.0103	350
1.0	4.9	0.0094	520
2.0	5.5	0.0095	580
3.0	4.5	----	---
4.0	3.7	0.0095	390
5.0	3.3	0.0110	300

The results show that as the amount of kaolin used is decreased below 1.0 gms. or increased above 2.0 gms., the yield and purity of enzyme fall. Since the results from the use of 1.0 and 2.0 gms. of kaolin are not significantly different, 1.0 gm. of kaolin/100 ml. of solution was chosen as the optimum amount.

4. The proper number of washings

The enzyme from three 90 ml. aliquots of dialysed culture filtrate, adjusted to pH 5.5 with 10 ml. of buffer, was adsorbed on 2.0 gms. of kaolin. The kaolin from the three samples was washed a varying number of times using 30 ml. of a 1:3 dilution of buffer at pH 5.5. The enzyme was eluted and dialysed as before.

As indicated by the results in Table VIII, washing aids in removing impurities, without decreasing the yield of enzyme.

5. Proper buffers to use in the washing and elution

The following solutions were made up for this experiment.

Wash solutions:

- a. McIlvaine buffer: a 1:3 dilution of buffer at pH 5.5.
- b. Acetate buffer: 2.72 gms. of NaOAc was dissolved in 400 ml. of water and the pH adjusted to pH 5.5 using 0.1 N HCl. The volume was then made up to 500 ml. (0.05 M.).

Eluting solutions:

- a. McIlvaine buffer, pH 8.0.
- b. "Tris" buffer, pH 8.14.

TABLE VIII

The Effect of Increasing the Number of Washings

No. of washings	microeq. NaOH used	$\frac{\text{mg. N}}{\text{ml.}}$	$\frac{\text{microeq. NaOH}}{\text{mg. N}}$
1	5.4	0.0140	390
2	5.5	0.0095	580
3	5.1	0.0072	710

c. Ammonia water, 0.15% as NH_3 .

To each of 8, 90 ml. aliquots of culture filtrate, adjusted to pH 5.5, was added 1.0 gm. of kaolin. The isolation of the enzyme was then carried out as before using different wash solutions and eluents. All eluents were dialysed overnight against running tap water. The enzymes were then assayed using the second formol titration method.

The results in Table IX, column "a" show that the wash solutions in order of decreasing usefulness are McIlvaine, acetate, and water, while that of the eluent are ammonia water, "tris," and McIlvaine buffers. However, as shown in column "b," after storing the enzymes a short period of time, the decrease in activity of the enzyme, obtained after using the McIlvaine buffer as the wash solution, is great enough to nullify its advantage over acetate buffer as a wash solution. This is not surprising since a phosphate is used as a salt in the McIlvaine buffer system. For this reason, acetate buffer was chosen as the wash solution and ammonia water as the eluent.

6. Percentage recovery

The following assay was performed on the results of a large-scale purification. In this experiment, McIlvaine buffers were used for the washing and elution. The volume of eluent was 7% that of the culture. The results are found in Table X.

TABLE IX

The Effect of Different Buffers as Wash and Eluting Solutions

Wash soln.	Eluting soln.	microeq. a*	NaOH used b**
McIlvaine	McIlvaine	4.20	---
water	McIlvaine	2.14	---
McIlvaine	"Tris"	5.72	2.36
Acetate	"Tris"	3.38	---
water	"Tris"	2.58	---
McIlvaine	NH ₄ OH	6.20	3.16
Acetate	NH ₄ OH	5.68	3.11
water	NH ₄ OH	3.48	---

* Time of incubation: 2.25 hours.

** Assay after storing enzymes at 4° C. for 24 hours.

Time of incubation: 1 hour.

TABLE X
Assay of Enzyme

Enzyme source	Dilution	microeq. NaOH used	$\frac{\text{mg. N}}{\text{ml.}}$
Undialysed media	---	---	0.720
Dialysed media	1.00	1.6	0.0100
	0.67	1.2	
	0.33	0.8	
Dialysed eluent	1.00	---	0.0059
	0.67	2.2	
	0.50	1.9	
	0.33	1.6	
	0.17	1.1	

The following calculations may be made from the results:

1. Since 0.33 ml. of eluent is equivalent to 1.0 ml. of dialysed filtrate, there is three times as much enzyme in the eluent. If a 100% yield were obtained, we would expect the eluent to be $\frac{1}{0.07}$ or 14 times as strong. Therefore, we can calculate $(3/14 \times 100)$ a 21% recovery of enzyme.

2. If we assume that the enzyme concentration for the undialysed filtrate is equal to that of the dialysed filtrate, we can perform the following calculation: 1 ml. eluent has 3 times the amount of enzyme as the culture filtrate and $\frac{0.0059}{0.72}$ or 0.0082 as much nitrogen. Therefore, we find that a $\frac{3.0}{0.0082}$ or 270-fold purification was obtained.

3. Using the values obtained for the dialysed culture filtrate and applying the same reasoning, we find that a $3.0 \times \frac{0.0100}{0.0057}$ or 5-fold purification was obtained over the dialysed filtrate.

7. Electrophoretic data

The enzyme used in the electrophoretic determination resulted from the purification as described on page 84.

Seventy-five mg. of dried enzyme was dissolved in 5.0 ml. of "tris" buffer, pH 7.6. The ionic strength was increased to 0.1 by the use of NaCl. The enzyme solution was dialysed for 18 hours against "tris" buffer of the same composition. Most of the brown color was removed by freezing the enzyme

solution, thawing, and removing the less colored supernatant liquid. The enzyme solution was then analysed by electrophoresis. The pictures, taken at 70 minutes and 141 minutes, are shown in Figs. 3a and 3b.

The content of the electrophoresis tube was separated into 5 components; the top and bottom of the ascending and descending limbs, and the U-tube, as indicated in Fig. 3b. Five drops of each were diluted with 5 ml. of H_2O and assayed by the second formol method, determining rate constants (a practice which will be described later). It should be stated, at this point, that there are two proteolytic enzymes isolated by adsorption on, and elution from, kaolin. One is heat resistant, while the other is heat labile. By heating the enzyme at $70^{\circ} C.$ for 0.5 hours, it is possible to destroy all the heat-labile enzyme without appreciably effecting the -stable enzyme. The result of the assay of the total of both enzymes is given in Graph IV and the result of the assay of the heat-stable one is given in Graph V.

The fact that we find both heat-labile and -stable enzymes in the active portions containing one boundary leads us to two explanations: either both enzymes move together at pH 7.6 to form one boundary peak, or that one is too dilute to show on the pattern. On examining the ratio of heat-stable enzyme to total enzyme (Graph V), we find that the order is: top of the ascending > bottom of the ascending = U-tube > bottom of the descending. This is the result one would expect if the

The Electrophoresis Pattern Using a "Tris" Buffer

at pH 7.4. $r/2 = 0.1$

Time: 70 mins.

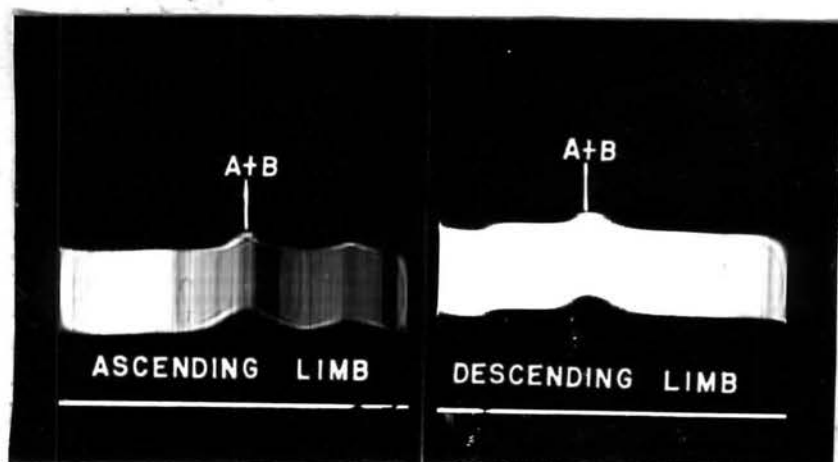


Fig. 3 a.

Time: 141 mins.

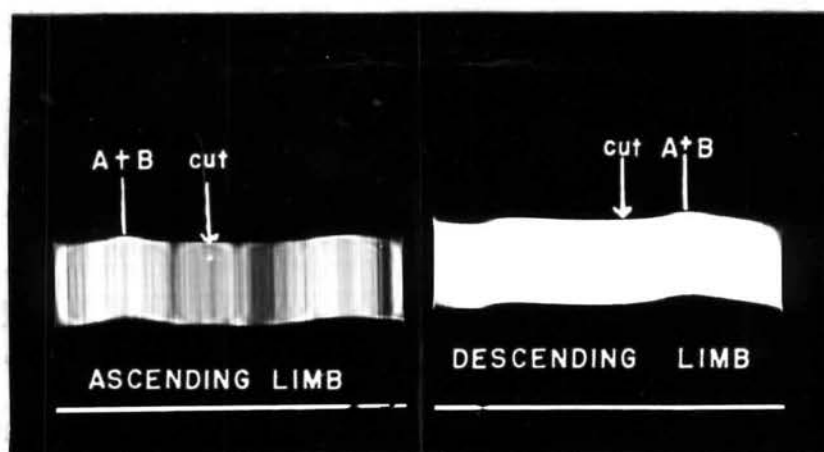
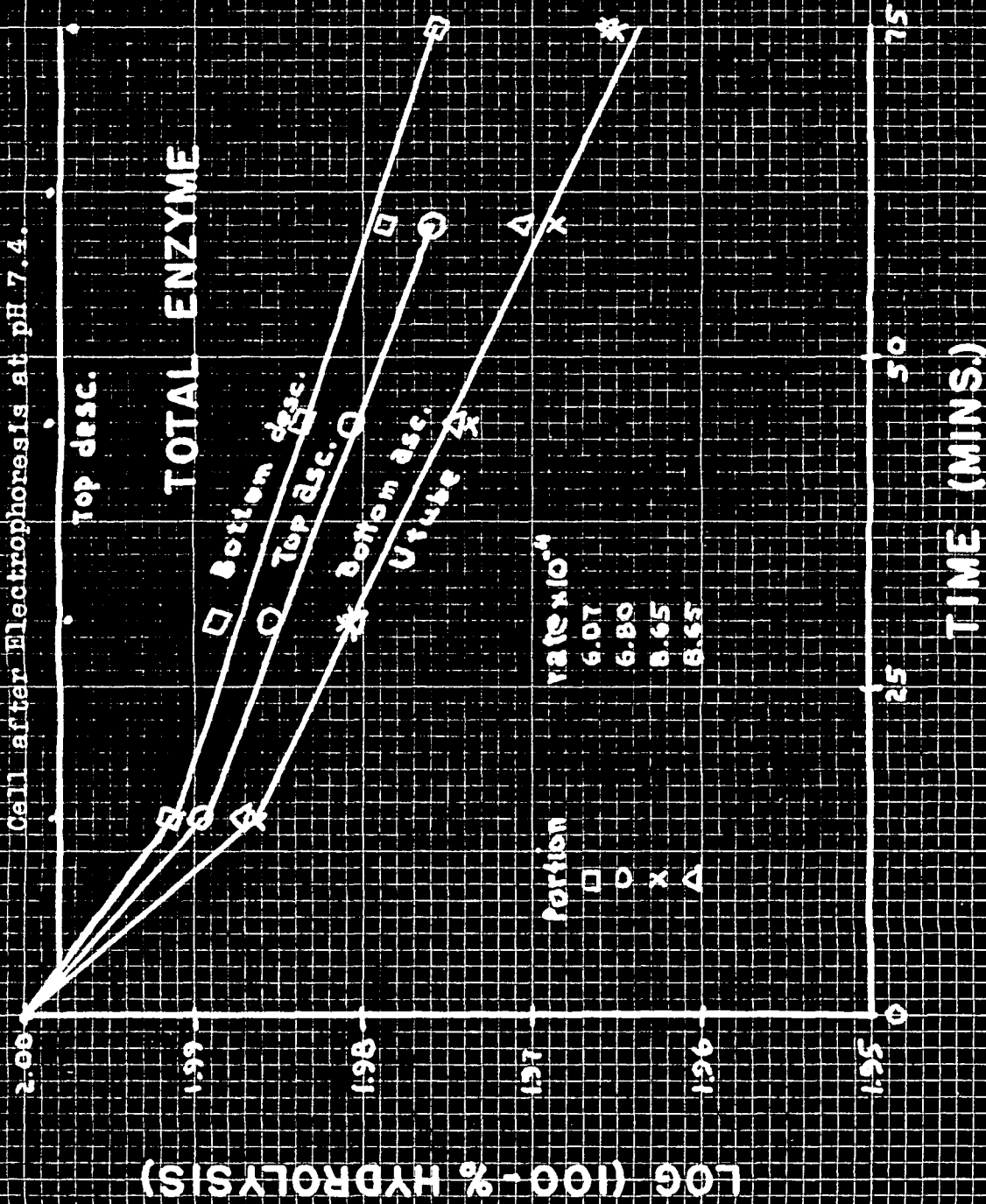


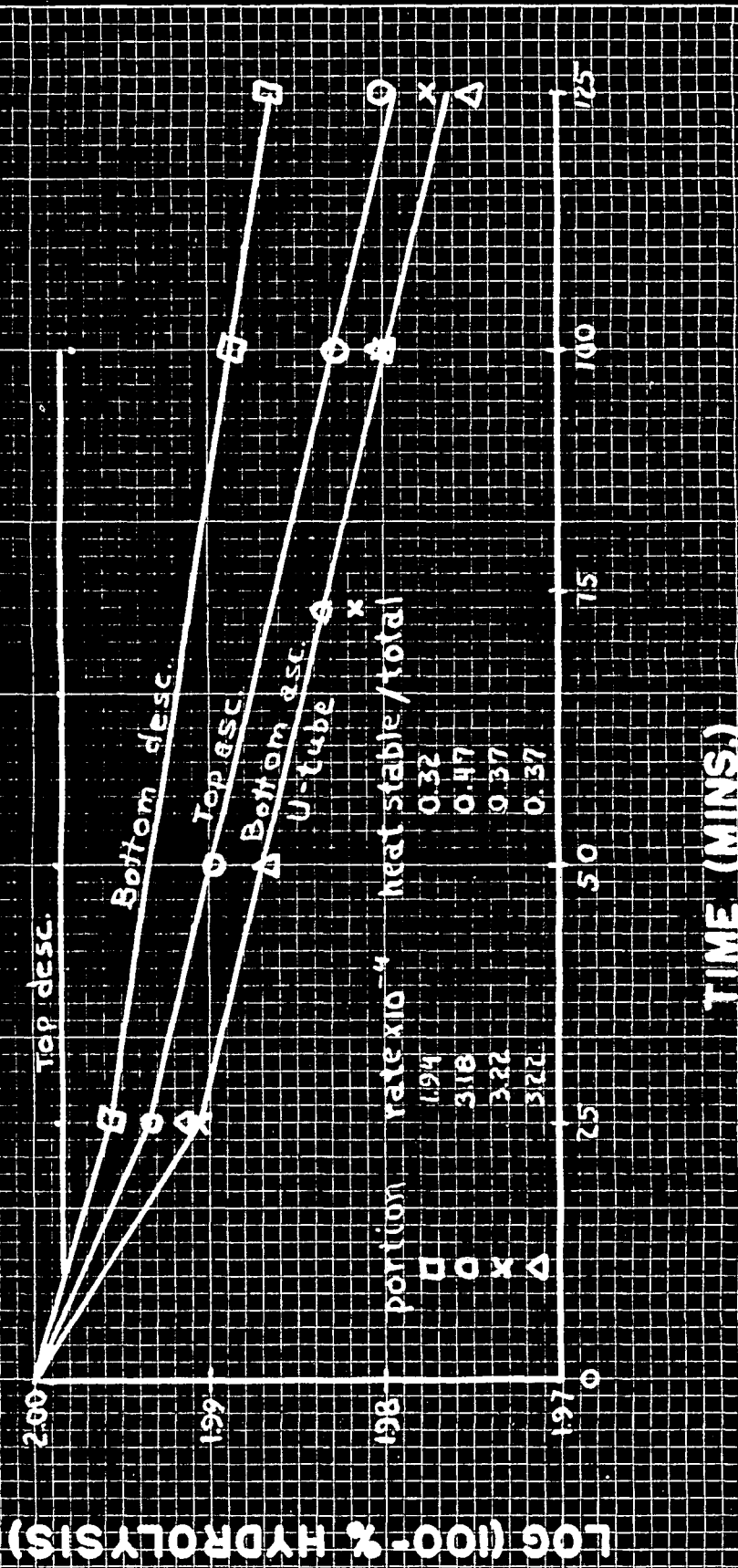
Fig. 3 b.

Graph IV. Assay of Total Enzyme from Various Portions of the Cell after Electrophoresis at pH 7.4.



Graph V. Assay of Heat-Stable Enzyme from Various Portions of the Cell after Electrophoresis at pH 7.4.

HEAT STABLE ENZYME



boundary peak consisted of both enzymes with the leading part of the boundary being the heat-resistant enzyme.

The fact that the top of the descending column has no enzyme is predictable.

Two electrophoresis patterns were made at pH 5.5 with an acetate buffer of 0.1 ionic strength. These are shown in Figs. 4 and 5. In Fig. 4, three distinct peaks are in evidence (A, B, and C). Upon assaying the various portions, it was found that there was no activity in the top of the ascending limb. In other words, peak "C" was not a proteolytic enzyme. The remaining portions were active. As shown in Fig. 5, a separation was made between peaks "A" and "B." The results of the assay of the 5 portions for both total enzyme and heat-stable enzyme (the activity after heating 30 minutes at 80° C.) are given in Table XI.

The following calculations can be made using the data in Table XI and Fig. 5:

1. Knowing that the length of the Tiselius cell is 50 mm. and that the pattern is 46 mm. in length, we may determine the length occupied by each section of the "cut." Next we can calculate the proportion of enzyme, represented by the two peaks, that is found in each section.

Section	Peak A	Peak B
Top asc.	9/30	0
Bottom asc.	1	15/20
Top desc.	0	12.5/18
Bottom desc.	28/32	1
U-tube	1	1

The Electrophoresis Pattern Using an Acetate Buffer

at pH 5.5. $r/2 = 0.1$

Time: 63 mins.

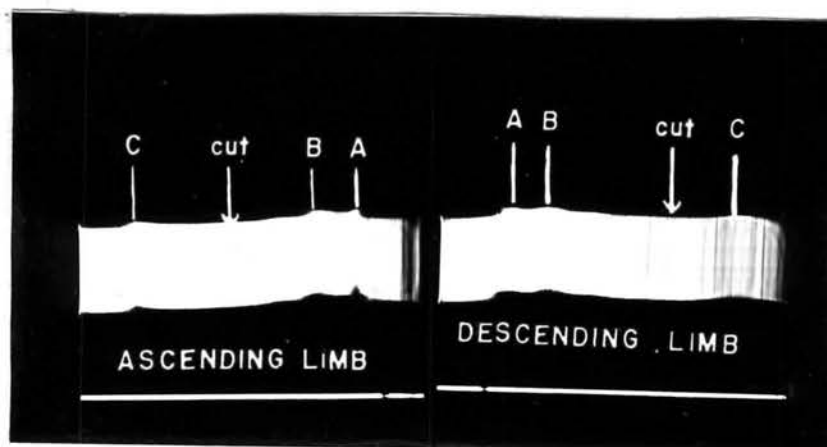


Fig. 4.

Time: 126 mins.

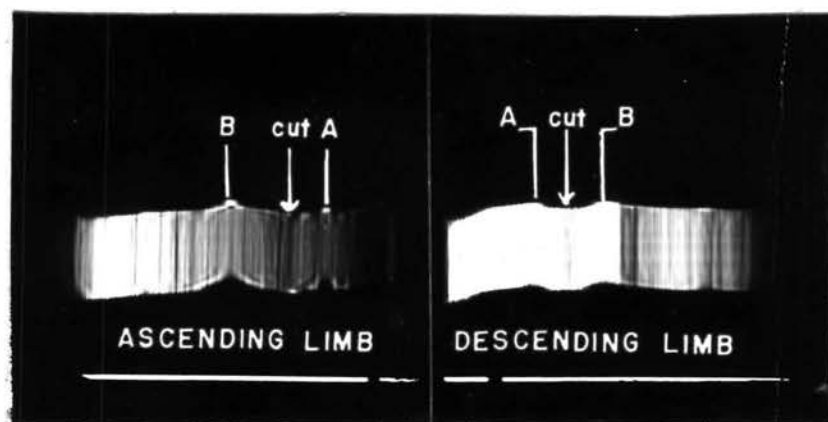


Fig. 5.

TABLE XI

Assay of Portions of Electrophoresis Cell
Corresponding to Fig. 5

Portion	Total enzyme	Heat-stable enzyme	<u>Heat-stable total</u>
Asc. top	0.735	0.215	0.29
Asc. bottom	2.48	0.67	0.27
Desc. top	0.125	0	0
Desc. bottom	1.29	0.32	0.25
U - tube	2.48	0.67	0.27

2. Let us assume that peak "A" is the heat-labile enzyme and peak "B," the heat-stable enzyme. Therefore, the top of the ascending limb contains only heat-stable enzyme and the top of the descending limb is made up of only the heat-labile enzyme. Knowing that the heat-stable enzyme occupies only 9/20 of the top of the ascending limb and that its rate constant is 0.735×10^{-3} , we can calculate a rate constant of 2.45×10^{-3} for this enzyme. In the same manner, a rate constant of 0.18×10^{-3} is obtained for the heat-labile enzyme. Also, from the ratio of heat-stable/total enzyme in the top of the ascending limb, it follows that 29% of the activity of the heat-stable enzyme is left after heating.

3. The calculated results may now be obtained for the remaining 3 portions:

Bottom of the ascending limb

$$\begin{aligned} \text{total enzyme} &= 2.45 \times 1.0 + 0.75 \times 0.18 = 2.59 \\ \text{heat-stable enzyme} &= 2.45 \times 0.29 = 0.71 \\ \text{heat-stable/total} &= 0.71/2.59 = 0.275 \end{aligned}$$

Bottom of descending limb

$$\begin{aligned} \text{total enzyme} &= 2.45 \times 0.875 + 0.18 \times 1.0 = 2.32 \\ \text{heat-stable enzyme} &= 2.45 \times 0.875 \times 0.29 = 0.62 \\ \text{heat-stable/total} &= 0.62/2.32 = 0.27 \end{aligned}$$

U-tube

$$\begin{aligned} \text{total enzyme} &= 2.45 + 0.18 = 2.63 \\ \text{heat-stable enzyme} &= 2.45 \times 0.29 = 0.71 \\ \text{heat-stable/total} &= 0.71/2.63 = 0.27 \end{aligned}$$

Although the calculated results for the bottom of the descending limb are double those obtained by assay, the figures check rather well. Therefore, we can say that peak

"B" is the boundary of the heat-stable enzyme and peak "A" is the boundary of the heat-labile enzyme.

A list of the mobilities of the various peaks is given in Table XII.

III. PROPERTIES OF THE PROTEOLYTIC ENZYME

A. Substrates Hydrolysed

Many authors have used gelatin as a substrate to demonstrate the proteolytic action of *P. vulgaris*. Jones (19) was the only investigator to use more than one substrate. He showed that casein and fibrin are also hydrolysed. In the course of our experimental work, several other proteins have been tested.

The following substrates were made up and assayed by the first formol titration method:

1. Raw tendon: Two very thin pieces of tendon, approximately 1 cm. in diameter, were placed in 5 ml. of 1:6 "tris" buffer, pH 7.4.
2. Autoclaved tendon: One piece of tendon, previously sterilized in the autoclave, was placed in 5 ml. of 1:6 "tris" buffer, pH 7.4.
3. Ribonucleic acid: A saturated solution of RNA was made in 0.01 N NaOH. Equal volumes of this solution and 1:6 "tris" buffer, pH 7.4, were mixed and adjusted to pH 7.4.
4. Desoxyribonucleic acid: This was made up as was the RNA except that 0.02 N NaOH was used to make a saturated solution.

TABLE XII

Mobilities of Enzymes at pH 5.5 and 7.4

Fig.	pH	Time (mins.)	Peak	Mobilities x 10 ⁻⁵ (Cm. ² /V. - sec.)	
				Ascending	Descending
3 a	7.4	70	A + B	- 4.84	- 4.55
3 b	7.4	141	A + B	- 4.80	- 4.48
4	5.5	63	A	- 1.78	- 1.10
	5.5	63	B	- 3.17	- 2.94
	5.5	63	C	- 11.9	- 12.1
5	5.5	126	A	- 1.23	- 1.00
	5.5	126	B	- 3.70	- 2.90

The results in Table XIII show that RNA is not hydrolysed, raw tendon and DNA are hydrolysed slightly, while autoclaved tendon is hydrolysed appreciably.

An experiment was performed to determine the optimum pH of enzyme activity when dried skimmed milk was used as a substrate. A 6% solution of Difco dried skimmed milk was divided into 30 ml. portions and adjusted to the pH values listed in Table XIV using 10% NaOH. Seven ml. portions of each plus 1 ml. of enzyme were assayed by the first formol titration method. The results show that, as with gelatin, pH 7.4 is optimum.

B. Activation and Inhibition of Enzyme

Cysteine and FeCl_2 have been used to activate the proteinases of the Clostridium group (17) (23) (25). Weil et al. (33) state that the proteinase of P. vulgaris was slightly activated by cysteine, slightly inactivated by Fe^{++} and greatly activated by a solution of both. In order to check these results, the experiment described next was performed. A solution of cysteine hydrochloride (20 mg./ml.), a solution of FeSO_4 (2.9 meq./ml.), and a 15% sodium citrate solution were made up. To tubes containing 4 ml. of gelatin substrate were added various combinations of these reagents. All solutions were adjusted to pH 7.4, 1 ml. of enzyme was added, and the first formol method was then performed.

The results given in Table XV show that cysteine and Fe^{++} inactivate the enzyme in the following decreasing order

TABLE XIII

The Effect of Enzyme on Different Substrates

Substrate	RNA	DNA	Raw tendon	Autoclaved tendon
microeq. NaOH used	0.0	0.4	0.4	4.3

TABLE XIV

The Effect of pH on the Hydrolysis of Casein

pH	6.8	7.0	7.2	7.4	7.6	7.8
microeq. NaOH used	0.9	1.3	1.4	1.6	1.3	0.6

TABLE XV
 The Effect of Ferrous Ions and Cysteine
 on Enzyme Activity

Fe ⁺⁺ (ml.)	Cysteine (ml.)	Citrate (ml.)	H ₂ O (ml.)	microeq. NaOH used
1	0	0	2	1.0
0	1	0	2	1.1
1	1	0	1	- 5.1*
0	0	1	2	2.5
1	0	1	1	1.4
0	1	1	1	0.9
1	1	1	0	1.7
0	0	0	3	2.7

* Precipitation in Absence of Citrate

of inactivation: cysteine, Fe^{++} , and cysteine - Fe^{++} . When citrate alone was added, there was no difference in comparison with the results when no solution was added. It will be noted that citrate must be present to prevent the precipitation of the cysteine - Fe^{++} mixture. (Weil used a citrate buffer.) These results differ greatly from those reported by Weil.

IV. ENZYME KINETICS

A. Stability Toward Heat

Jones (19) stated in his article that the proteolytic enzyme of P. vulgaris was not destroyed until it was boiled for 15 minutes.

In order to determine the thermal inactivation rate of the enzyme, a series of experiments was performed by heating 1.2 ml. aliquots of enzyme for various intervals of time and then analysing 1 ml. of each by means of the first formol titration method. The log of microeq. of NaOH used was plotted against time of heating. This experiment was repeated, heating the enzyme at different temperatures. The results will be found in Graph VI.

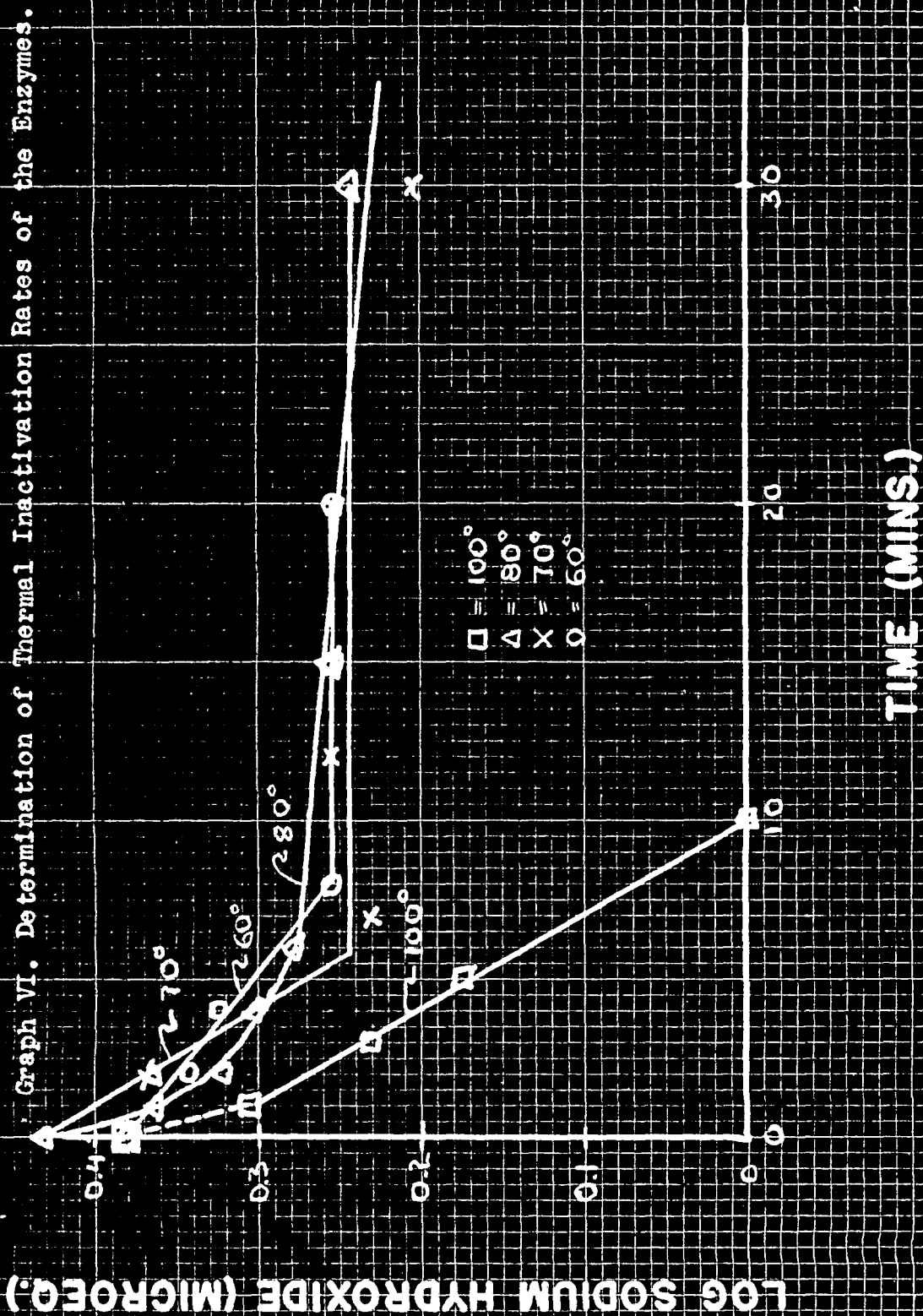
From the results in Graph VI, it was concluded that there are two enzymes present in the preparations derived by the adsorption-elution technique. One is only slightly inactivated at a temperature of 80° C. (This is called the heat-stable enzyme.) The other enzyme is destroyed quite rapidly at 60° C. (This is called the heat-labile enzyme.)

Graph VI. Determination of Thermal Inactivation Rates of the Enzymes.

LOG SODIUM HYDROXIDE (MICROEQ.)

TIME (MINS.)

□ = 100°
△ = 80°
X = 70°
○ = 60°



B. Relationship of enzyme-substrate concentrations

The remainder of the work was done using the second formol method.

In order to ascertain the per cent hydrolysis represented by each microeq. of NaOH used, the following experiments were performed: 10 ml. of a strong enzyme solution were added to each of three gelatin solutions which, when made up to a final volume of 32 ml., represented 1%, 2%, and 3% gelatin solutions as made up for assay. These were incubated 18 hours at 37° C. Portions of these solutions were diluted with water to represent the amount of end products formed by varying percentages of hydrolysis of the original substrate. These were titrated in the usual manner (Column a, Table XVI). A titration value for the unhydrolysed gelatin was then obtained and from this value was calculated the titration value of the unhydrolysed gelatin remaining after the partial hydrolysis (Column b). By adding the values in Columns a and b, the total value, representing the titration value for end products plus unhydrolysed gelatin, was obtained (Column c). If the value for the unhydrolysed gelatin is then subtracted from the total, the value for the milliliters of NaOH used is obtained which can then be converted into microeqs. of NaOH (Column d). These values were then plotted (Graph VII).

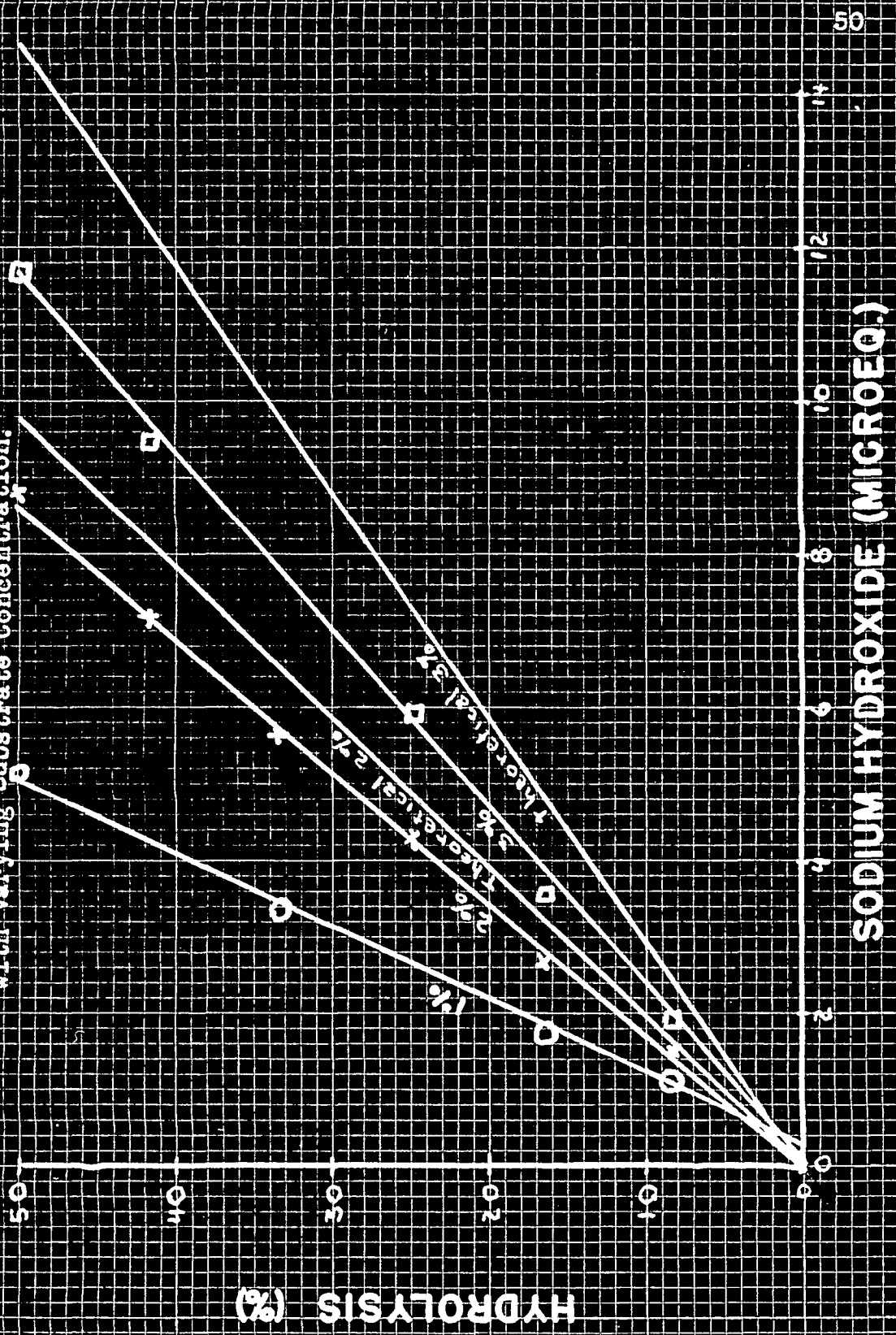
Graph VII shows that the values obtained with 2% and 3% gelatin do not check with those calculated assuming that the 1% gelatin was 100% hydrolysed. In order to determine

TABLE XVI

The Relation Between Theoretical % Hydrolysis and
Amount of Sodium Hydroxide Used

% gel.	theoretical % hydrolysis	microeq. end products (a)	microeq. substrate (b)	microeq. total	microeq. NaOH used
1	0	0	5.56	5.56	0
	8.34	1.55	5.08	6.63	1.07
	16.7	2.73	4.58	7.31	1.75
	33.3	5.22	3.71	8.93	3.37
	50.0	7.93	2.78	10.71	5.15
2	0	0	10.32	10.23	0
	8.34	2.42	9.47	11.89	1.57
	16.7	4.52	8.60	13.12	2.80
	25.0	6.93	7.75	14.68	4.36
	33.3	9.08	6.88	15.96	5.64
	41.7	11.45	6.02	17.47	7.15
	50.0	13.90	5.17	19.07	8.75
3	0	0	15.88	15.88	0
	8.34	3.26	14.54	17.80	1.92
	16.7	6.20	13.23	19.43	3.55
	25.0	9.85	11.91	21.76	5.88
	41.7	16.10	9.25	25.35	9.47
	50.0	19.50	7.93	27.43	11.55

Graph VII. Correlation Between % Hydrolysis and microeq. of NaOH used with Varying Substrate Concentration.



the actual amount of hydrolysis in the original samples after incubation, the biuret reaction was employed. The results are given in Table XVII. By multiplying the slopes of the lines in Graph VII by the per cent hydrolysis actually found, we can calculate the per cent hydrolysis represented by each microeq. of NaOH used. These results are found in Table XVIII. If we then multiply the % hydrolysis/microeq. NaOH by the per cent gelatin, we obtain values of 5.2, 4.8, and 5.0 for 1%, 2%, and 3% gelatin respectively. The average of these numbers is 5.0. Using this value as a basis, we can now calculate the % hydrolysis/microeq. NaOH for each per cent substrate used by the formula $5.0/\%$ substrate.

Using the above figures, one can calculate that gelatin contains 20 microeq. of peptide bonds for each per cent concentration of gelatin as assayed.

An experiment was performed determining the rate of several dilutions of an enzyme preparation with 1% gelatin as the substrate. This experiment was repeated with other concentrations of gelatin, all made from the 6% gelatin "tris" buffer solution described on pg. 13. The results are given in Graphs VIII through XII.

It will be seen from these graphs that a plot of the log of the per cent unhydrolysed substrate against time gives the curve for a typical first order reaction.

If the enzyme concentration is plotted against the rate constant for a first order reaction, Graph XIII is obtained.

TABLE XVII

The Determination of % Hydrolysis

Hydrolysed gel. (%)	Dilution tested (% gel.)	Opt. density	% gel. unhydrolysed	% hydrolysis
3	.254	.155	.156	39
2	.333	.194	.196	41
1	.417	.208	.210	50
unhydrolysed	.200	.198	0	0

TABLE XVIII

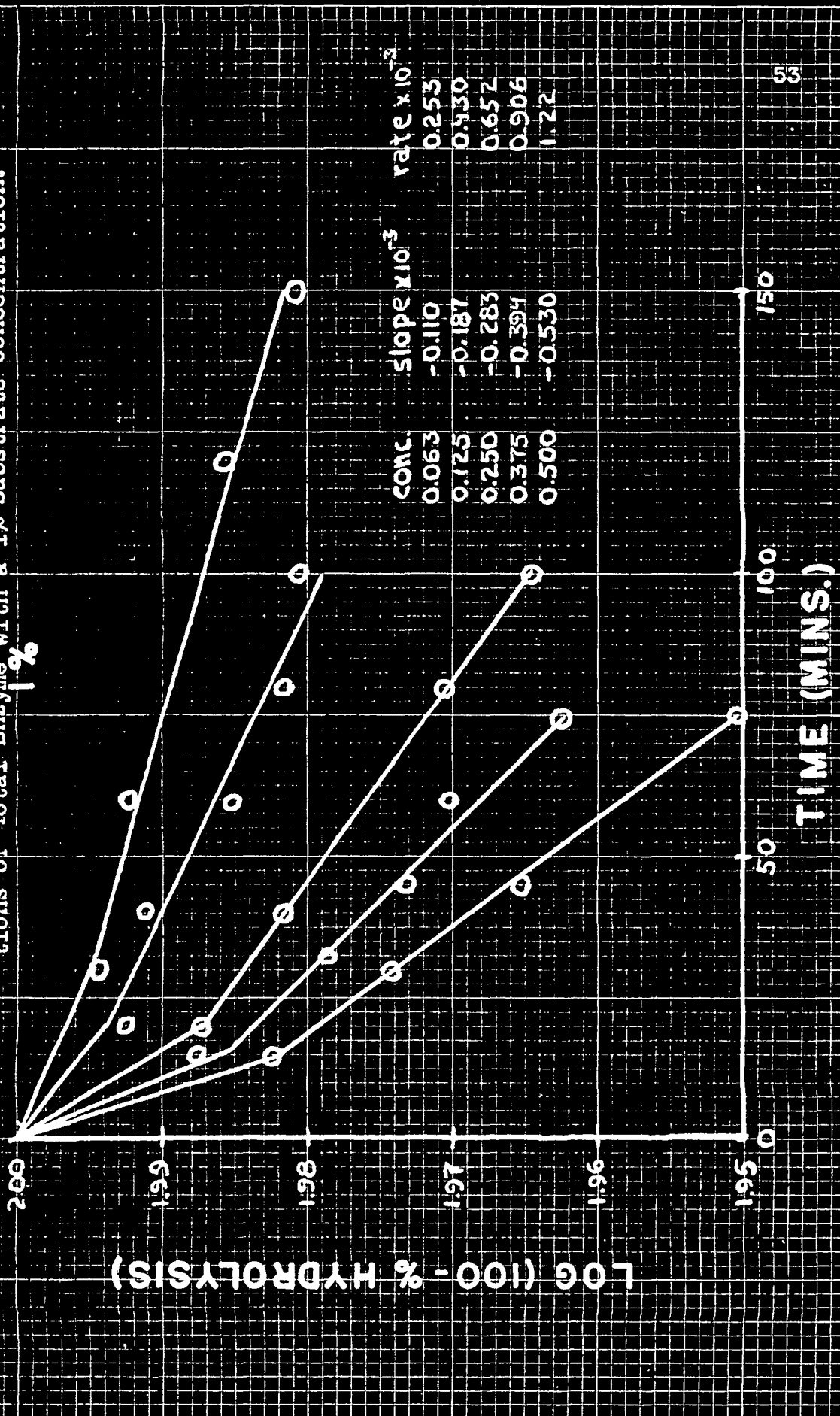
The Relationship Between True % Hydrolysis
and Amount of Sodium Hydroxide Used

% gel.	<u>% hydrolysis</u> microeq. *	% hydrolysis**	<u>% hydrolysis</u> microeq. NaOH
1	10.4	50	5.2
2	5.7	41	2.4
3	4.5	39	1.7

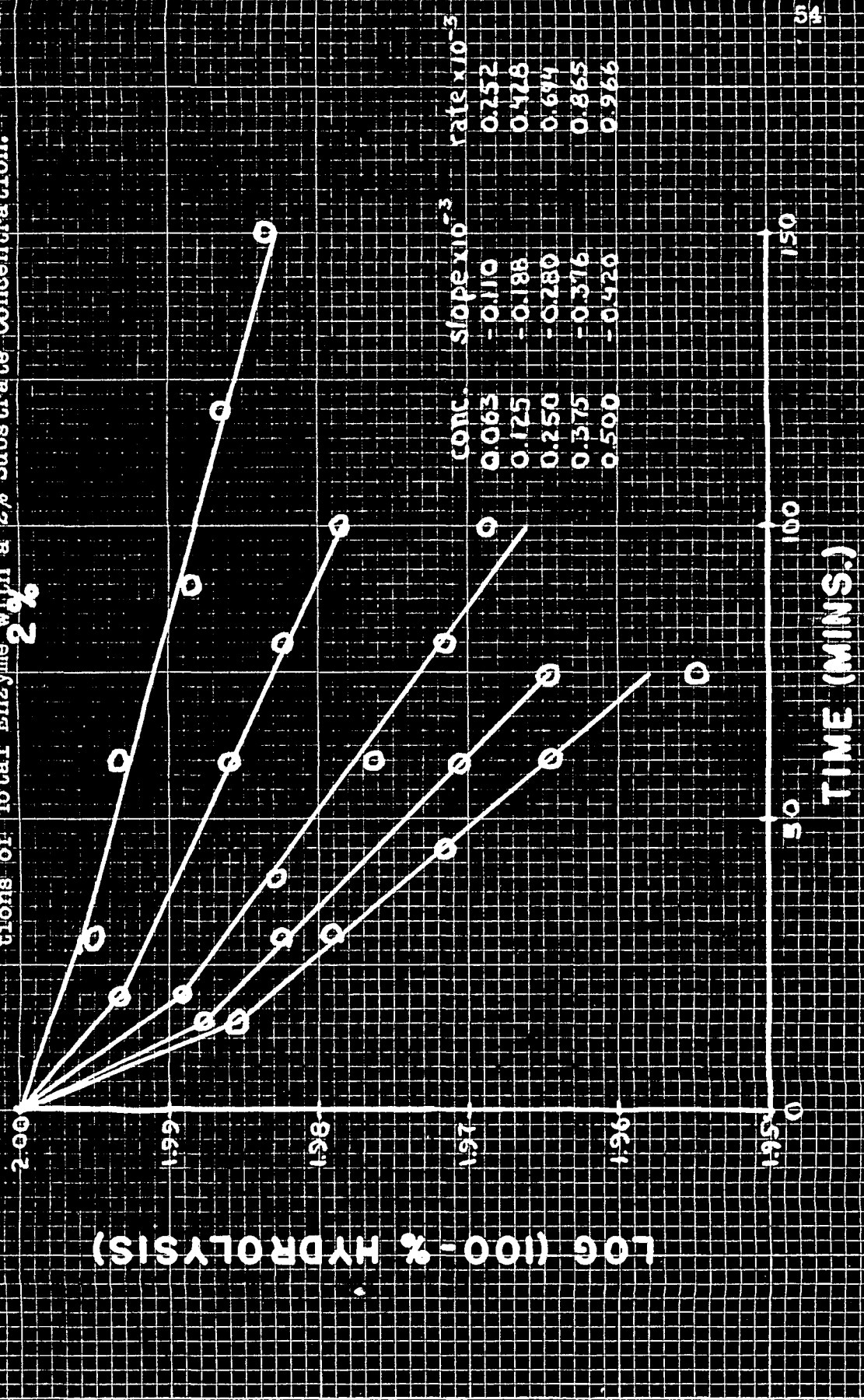
* From Graph VII

** From Table XVII

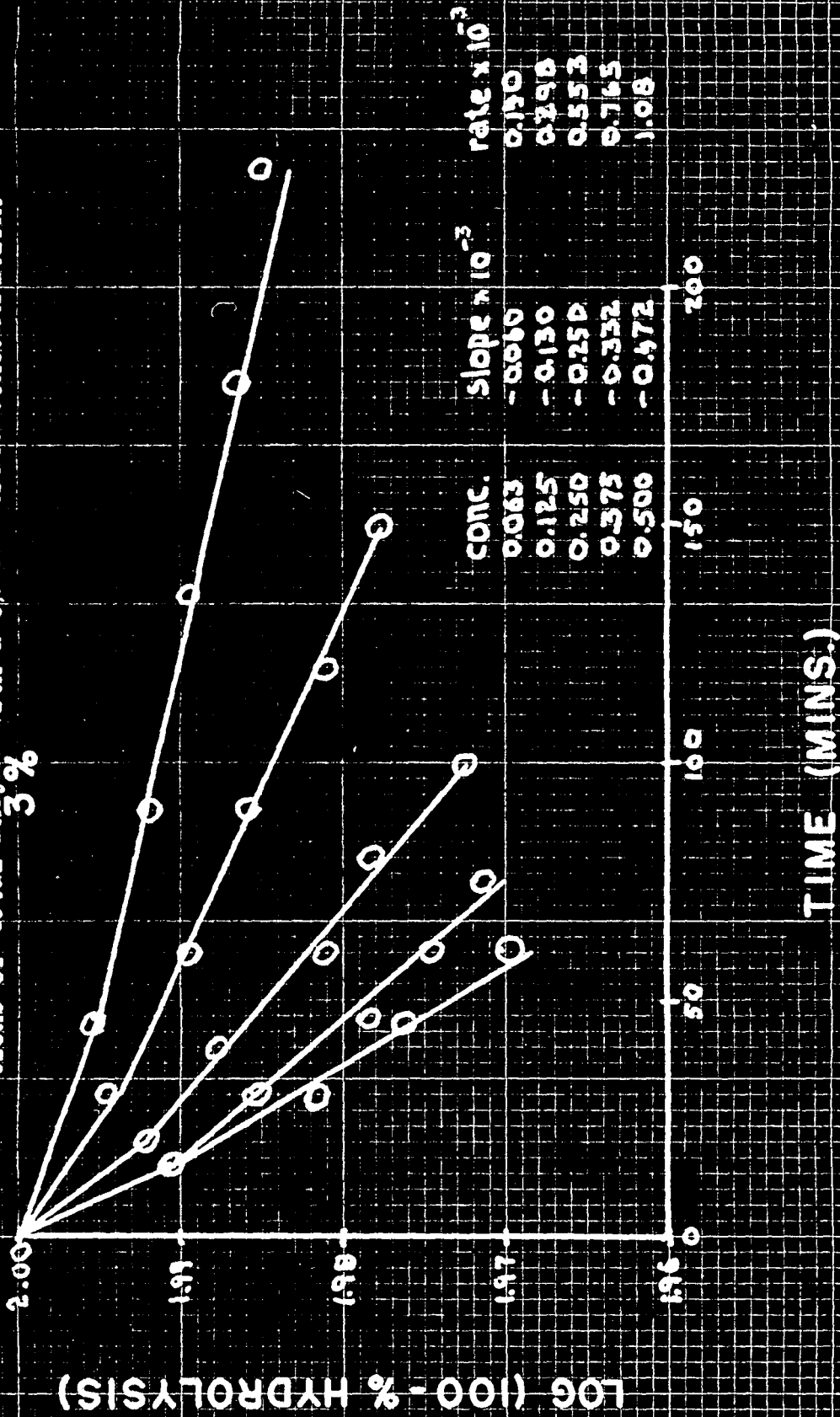
Graph VIII. Determination of Rate Constants for Different Concentrations of Total Enzyme with a 1% Substrate Concentration.



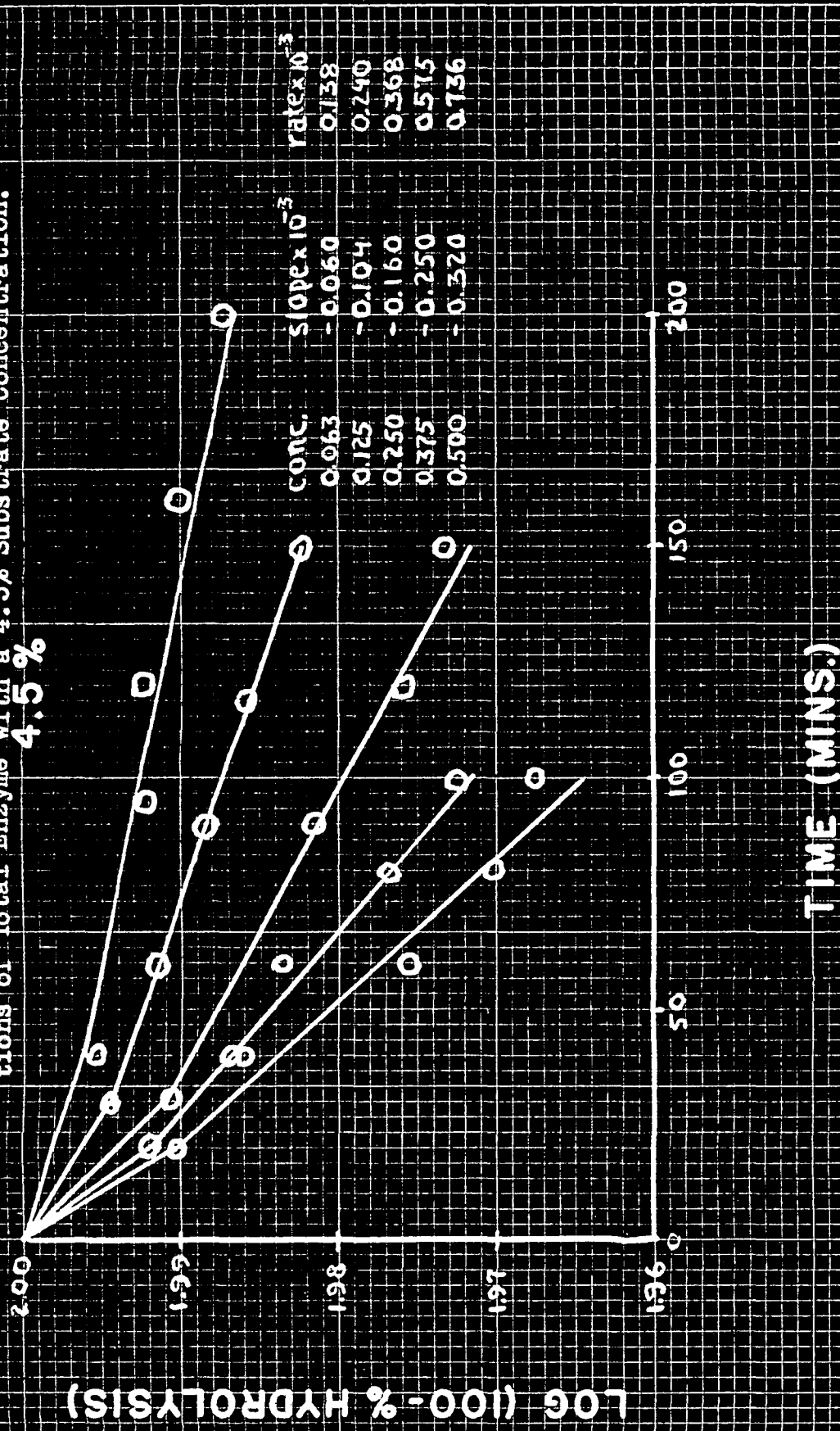
Graph IX. Determination of Rate Constants for Different Concentrations of Total Enzyme with a 2% Substrate Concentration.



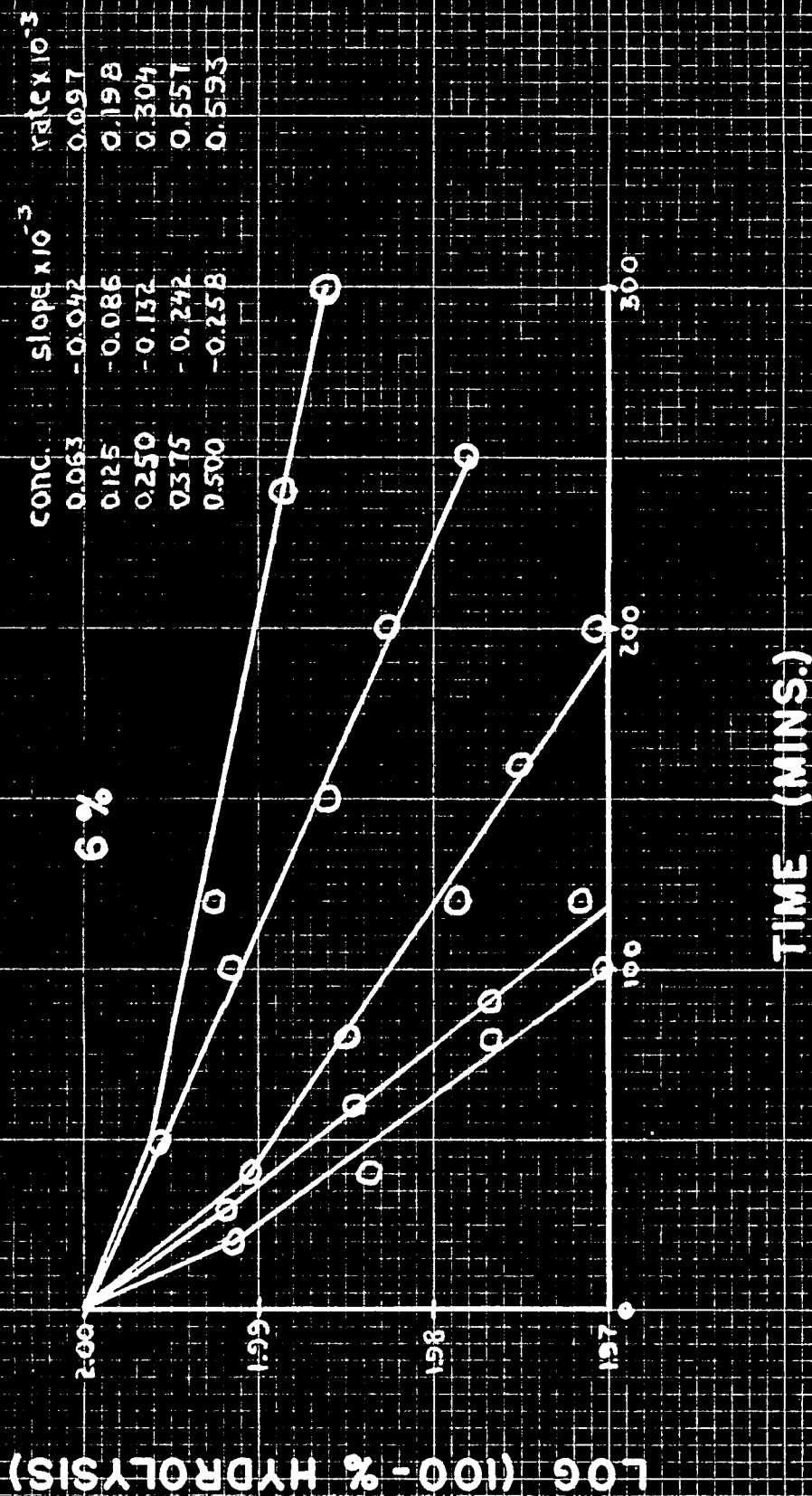
Graph X. Determination of Rate Constants for Different Concentrations of Total Enzyme with a 3% Substrate Concentration.



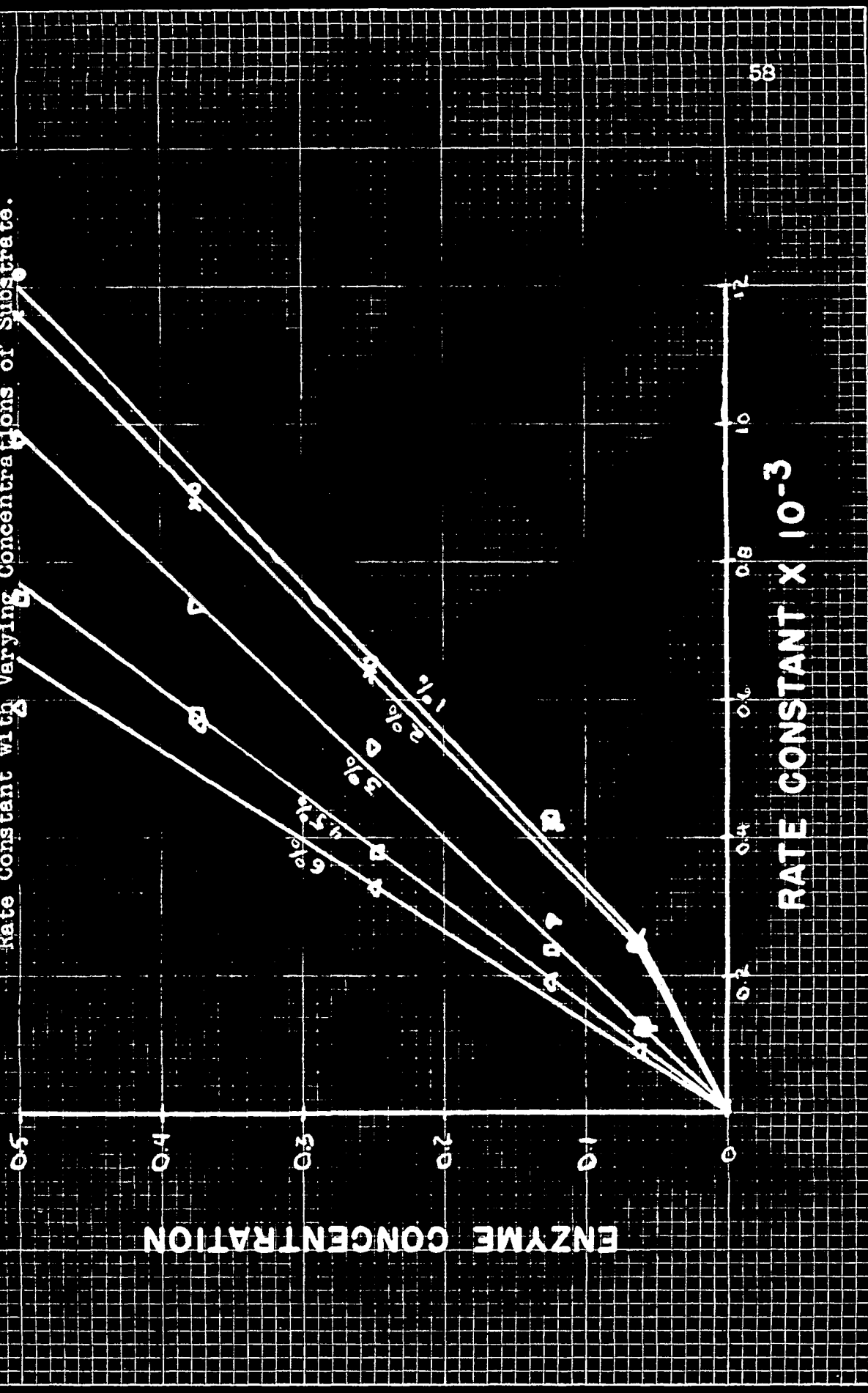
Graph XI. Determination of Rate Constants for Different Concentrations of Total Enzyme with a 4.5% Substrate Concentration.



Graph XII. Determination of Rate Constants for Different Concentrations of Total Enzyme with a 6% Substrate Concentration.



Graph XIII. The Correlation Between Concentration of Total Enzymes and Rate Constant with Varying Concentrations of Substrate.



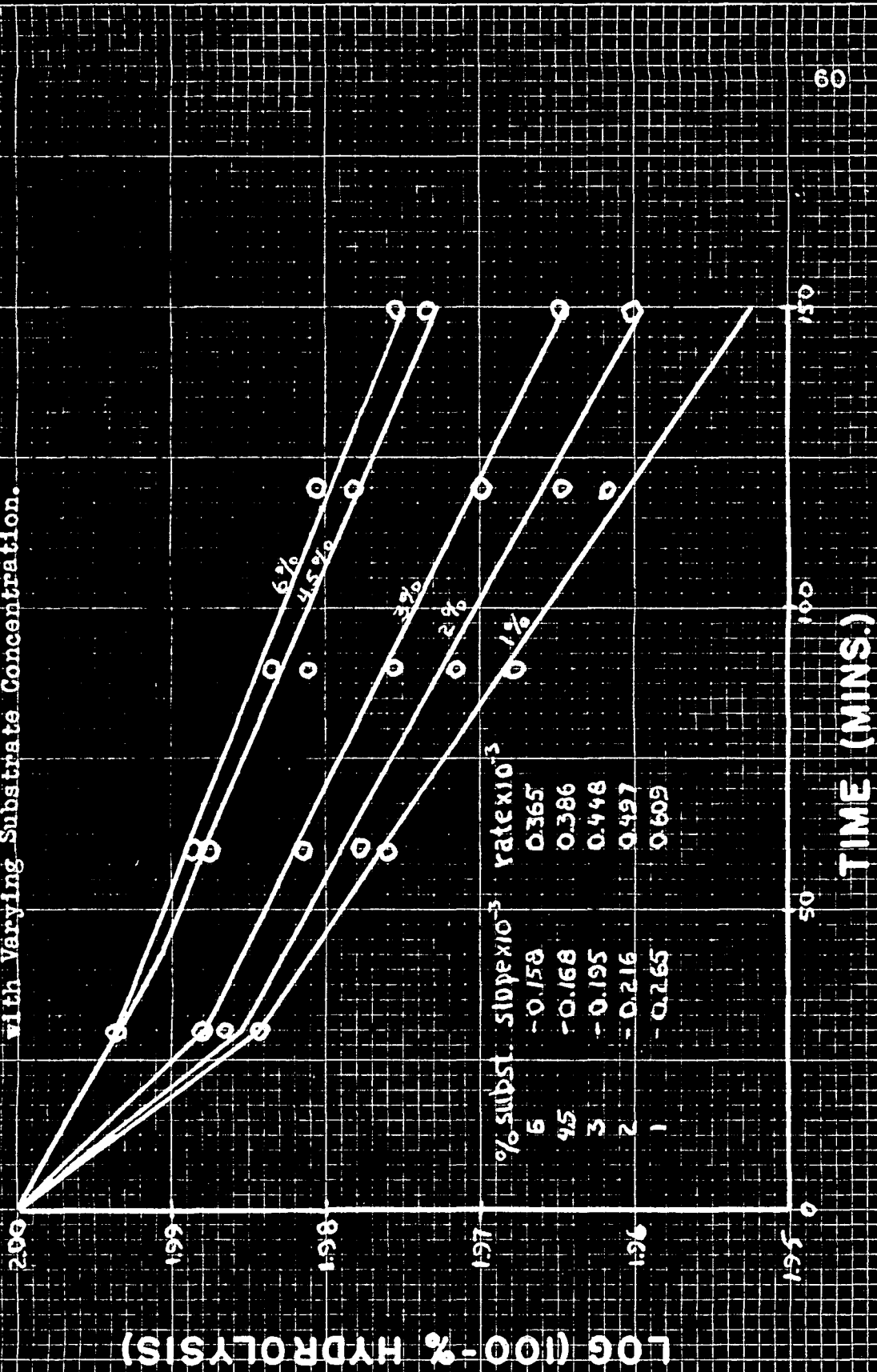
When 1% and 2% gelatin solutions are used, there is no relation between enzyme concentration and the rate constant. However, when a 3%, 4.5%, or 6% gelatin substrate is used, the relationship is a direct proportion. Since this is true, a unit of enzyme activity may now be established: one unit of enzyme activity is defined as that enzyme concentration which gives a rate constant of 0.0001 when determined at 37° C. by the second formol method using a 3% gelatin-"tris" buffer solution as substrate.

Another experiment was performed using a 0.25 dilution of one enzyme preparation and varying the concentration of gelatin used. A plot of log per cent unhydrolysed substrate against time is given in Graph XIV. Now, if a correction factor for all concentrations of enzyme given as 0.25 is applied so that the rates given for this concentration in Graphs VIII through XII is the same as for the 0.25 concentration in Graph XIV, we can adjust all the values so that all concentrations are equivalent. If we plot per cent gelatin against rate constants for the various dilutions of enzyme, the plots in Graph XV are obtained.

It is evident, from Graph XV, that there is a definite amount of substrate inhibition.

All of the above experiments were repeated using the heat-stable enzyme remaining after heating both enzymes for 2 minutes at 100° C. A plot of enzyme concentration vs. rate constants is found in Graph XVI. The enzyme concentrations

Graph XIV. The Determination of Rate Constants of Total Enzyme with Varying Substrate Concentration.



LOG (100 - % HYDROLYSIS)

TIME (MINS.)

60

150

100

50

195

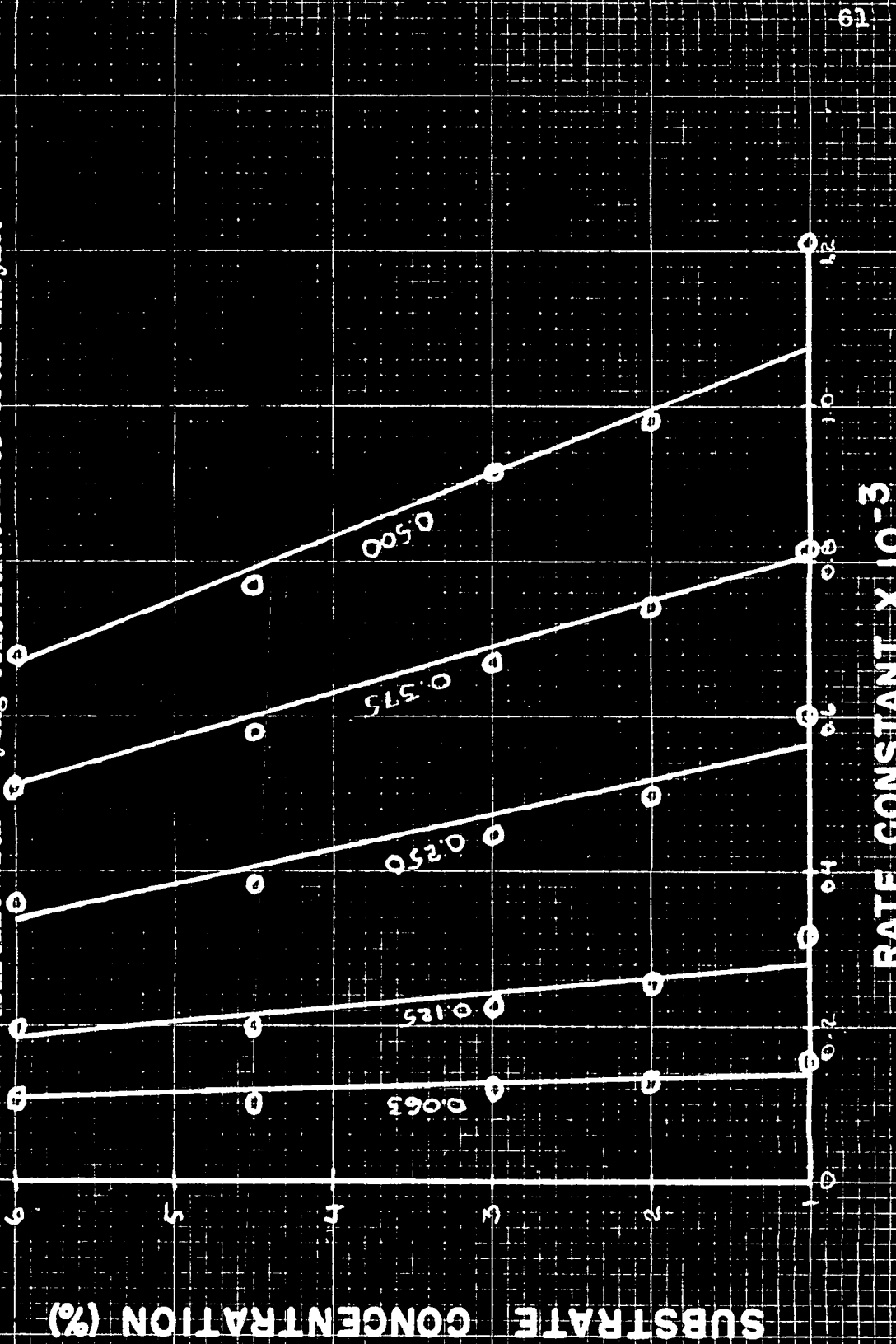
197

198

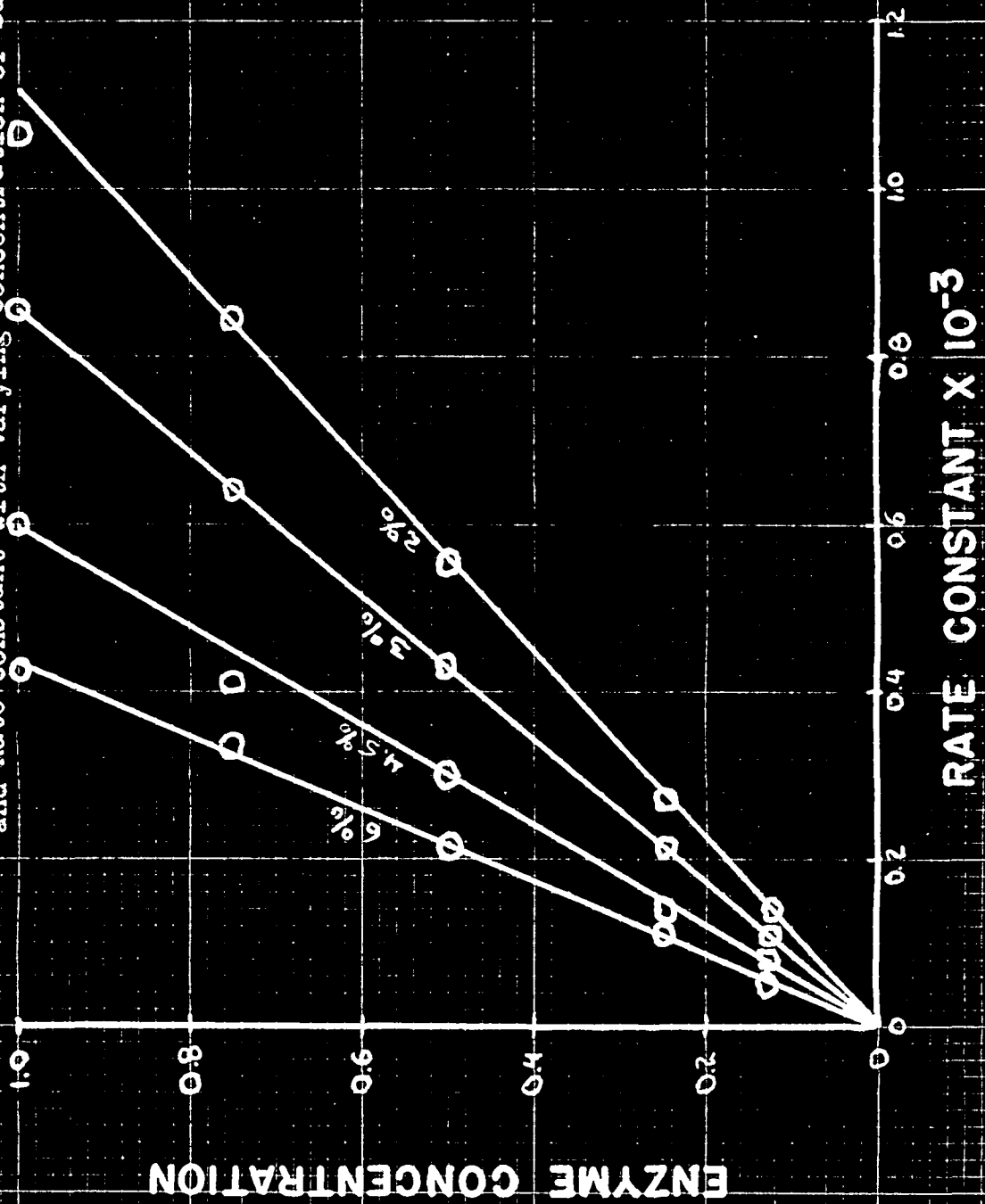
199

200

Graph XV. The Correlation Between Substrate Concentration and Rate Constant with Varying Concentration of Total Enzyme.

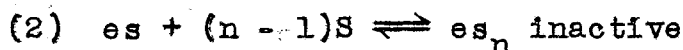


Graph XVI. The Correlation Between Concentration of Heat-Stable Enzyme and Rate Constant with Varying Concentration of Substrate.



were then adjusted in the manner described previously. A plot of substrate concentration vs. rate constants at five different enzyme concentrations is shown in Graph XVII. Again, it is evident that there is substrate inhibition.

In the case of the simpler system, heat-stable enzyme alone, we may write the following equations to explain the kinetics of the reaction (12):



If the rate of product formation in equation (1) is dependent on the concentration of es , then:

$$(3) \quad \frac{(e)(S)}{(es)} = K_1 \text{ and}$$

$$(4) \quad \frac{(es)(S^{n-1})}{(es_n)} = K_2$$

Let $es = p$ and $es_n = q$. Then, from (3) and (4), we obtain:

$$(5) \quad (e - p - q)S = pK_1 \text{ and}$$

$$(6) \quad pS^{n-1} = qK_2$$

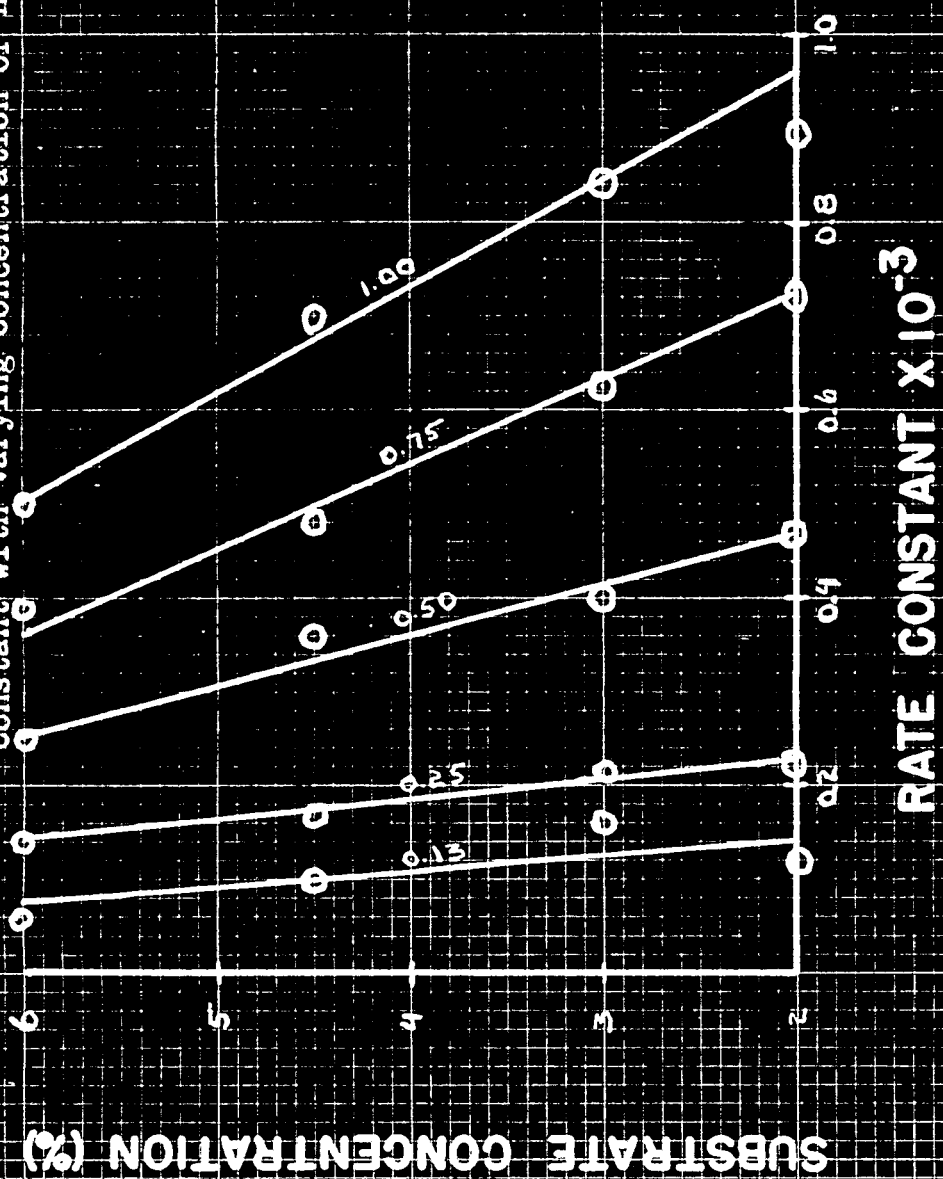
From (5) and (6):

$$(7) \quad q = \frac{eS - pS - pK_1}{S} = \frac{pS^{n-1}}{K_2}$$

By solving for p , and since $\frac{dp}{dt} = V = kp$, we obtain

$$(8) \quad V = \frac{keS}{S + K_1 + \frac{S^n}{K_2}} = kp$$

Graph XVII. The Correlation Between Substrate Concentration and Rate Constant with Varying Concentration of Heat-Stable Enzyme.



Since the concentration of e is unknown, k_e can be expressed as V_m , the maximum velocity. Then, by mathematical manipulation:

$$(9) \quad \frac{S}{V} = \frac{K_1}{V_m} + \frac{1}{V_m} \left(S + \frac{S^n}{K_2} \right).$$

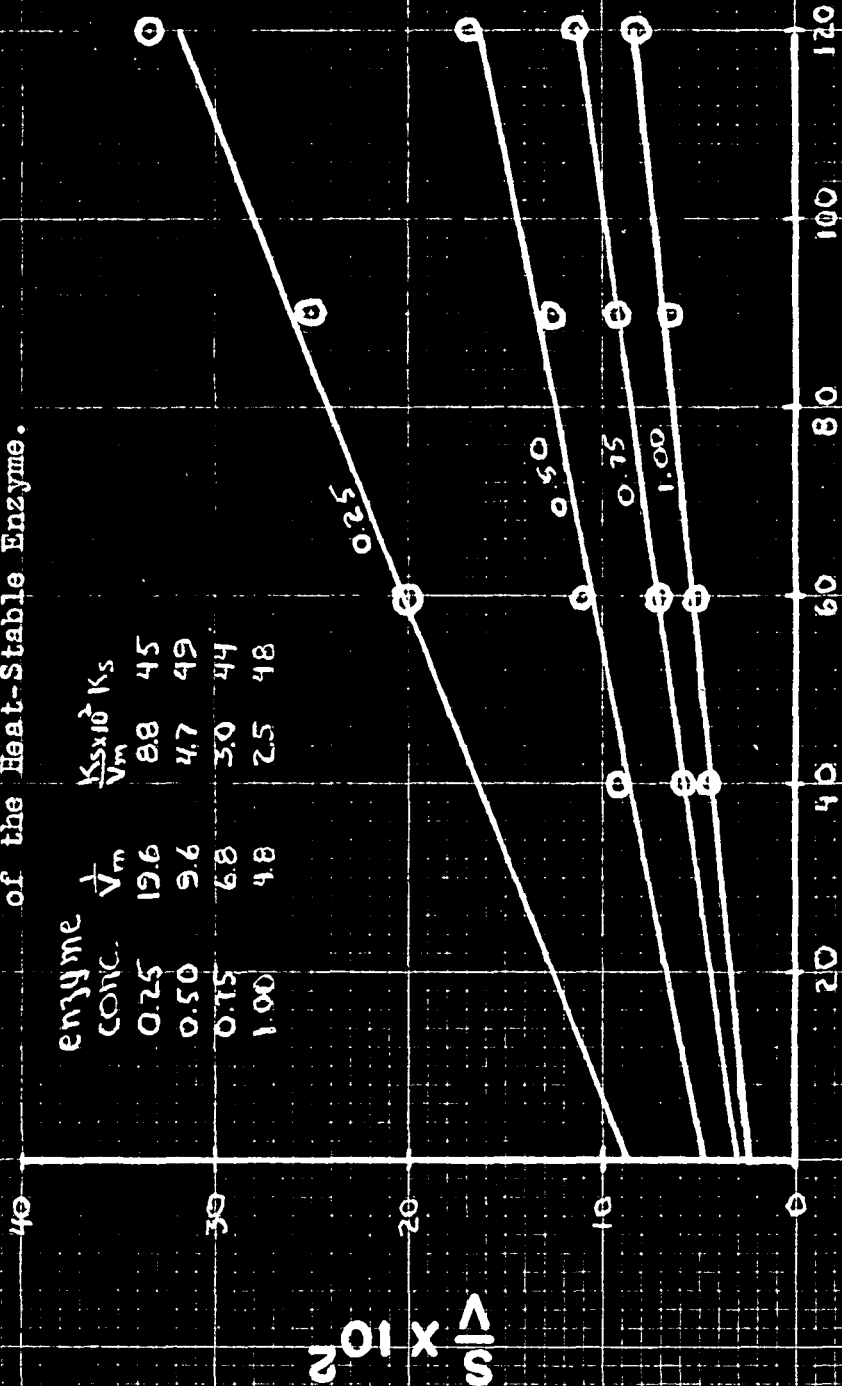
It will be seen from equation (9) that a plot of $\frac{S}{V}$ vs. S will give a straight line, when $\frac{S^n}{K_2}$ is negligible, with a slope of $\frac{1}{V_m}$ and an intercept of $\frac{K_1}{V_m}$ at $S = 0$. The value $\frac{S^n}{K_2}$ is negligible for small values of S . Thus, the equation reduces to the typical Michaelis-Menten expression.

In order to substitute the values given in Graph XVIII into this equation, the values for the rate constants must be converted to velocity constants. Graph XVIII is obtained by plotting the Michaelis-Menten equation using the velocity constants for the heat-stable enzyme. The values for the Michaelis-Menten constant, K_s , are obtained by dividing the intercepts by the slopes.

Before it is possible to determine K_s for the heat-labile enzyme, it is necessary to know how much of the heat-stable enzyme is inactivated when the total enzyme is heated to eliminate the heat-labile enzyme. The amount of inactivation was determined by heating a sample of the mixture at 100°C . for 1 minute to inactivate the heat-labile enzyme. Determinations of activity were made before and after reheating at

Graph XVIII. The Determination of the Michaelis-Menten Constant of the Heat-Stable Enzyme.

enzyme conc.	V_m	$K_s \times 10^3$
0.25	19.6	8.8
0.50	9.6	4.7
0.75	6.8	3.0
1.00	4.8	2.5



S (MICROEQS.)

100° C. for 2 minutes. It was found that 56% of the heat-stable enzyme remained. If we double the rate constants for the total enzyme (enzyme concentrations in Graph XV are 1/2 those in Graph XVII) and divide the rate constants for the heat-stable enzyme by 0.56, and then convert these values into velocity constants, we can obtain the velocity constants for the heat-labile enzyme by subtraction. The substitution of these values into the Michaelis-Menten equation gives the results shown in Graph XIX.

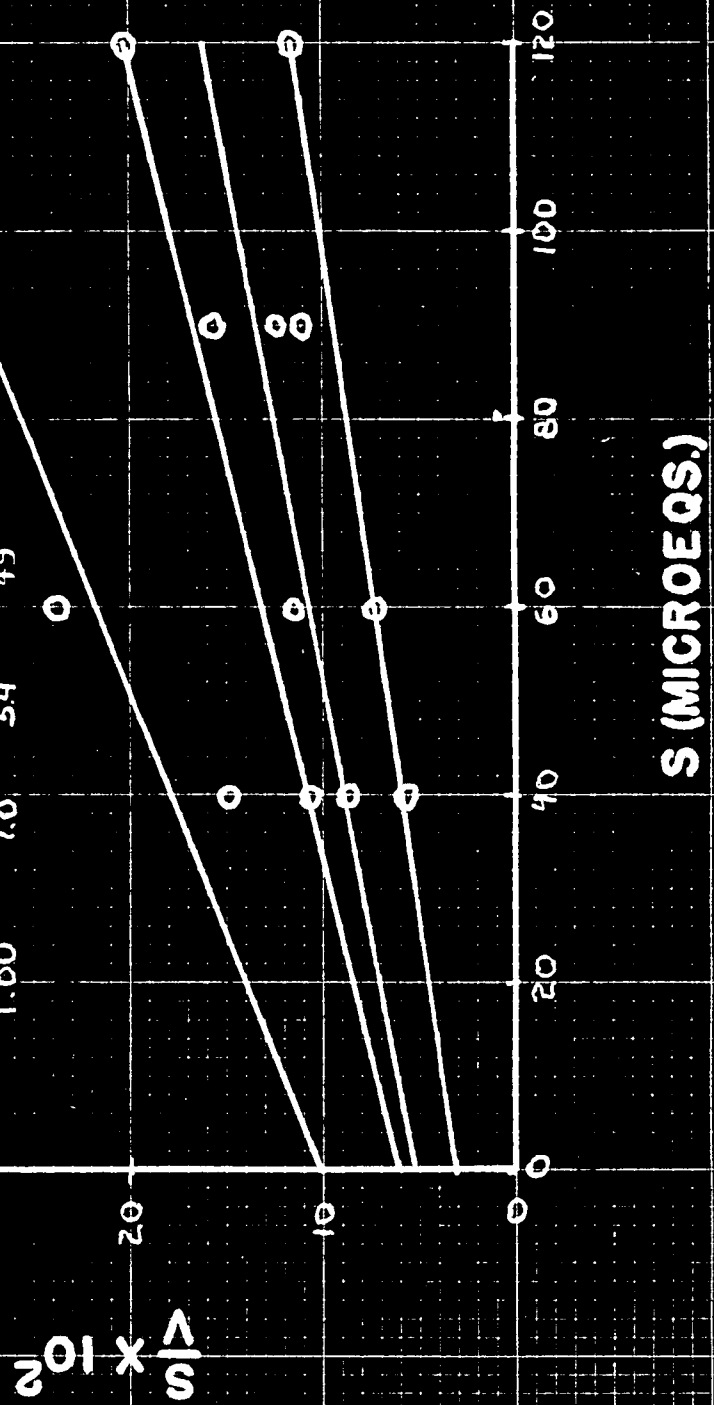
Thus, K_s for both enzymes is about 50.

It can be shown that the velocity constants for the heat-labile enzyme divided by the concentration of substrate gives values which increase as substrate concentration increases. The reverse is true for the heat-stable enzyme. This shows that the heat-stable enzyme is inhibited by the substrate. The heat-labile enzyme may or may not be inhibited by the substrate.

Inspection of Graphs VIII through XII shows that the first order curves of $\log (100 - \% \text{ hydrolysis})$ vs. time do not extrapolate back to 0% hydrolysis at zero time. Also, it will be noted that as the enzyme concentration increases, the apparent initial substrate concentration decreases. To explain this, one may postulate a rapid initial rate of hydrolysis which becomes first order before the first sample can be analysed. Because gelatin is a heterogeneous mixture, there was the possibility that different fractions hydrolysed at different

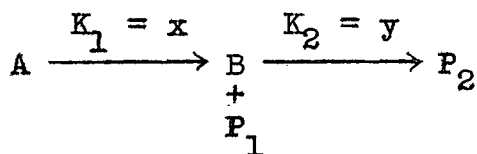
Graph XIX. The Determination of the Michaelis-Menten Constant of the Heat-Labile Enzyme.

enzyme CONC.	$\frac{1}{V_m}$	$\frac{K_s \times 10^2}{V_m}$	K_s
0.25	19.3	102	53
0.50	11.9	6.0	50
0.75	9.3	5.1	55
1.00	7.0	3.4	49



rates. The next experiment was performed to determine whether the different sizes of the molecules had any effect. A 6% solution of substrate was dialysed 2 days against tap water. The pH was readjusted to 7.4 and the "tris" buffer was replaced so that a 3% solution of dialysed gelatin was comparable to a 3% solution of undialysed substrate. The results of the hydrolysis of both by the same enzyme preparation shows (Graph XX) that dialysis decreases the apparent initial substrate concentration. In other words, the larger molecules seem to be hydrolysed faster, thus giving a very rapid initial rate.

To explain the above effect mathematically, one can assume the hydrolysis of a large molecule results in the formation of a smaller molecule plus some amount of product. The smaller molecule is then hydrolysed to form additional product. (This, unquestionably, is an oversimplification of what actually takes place.) This sequence of events may be represented as follows:

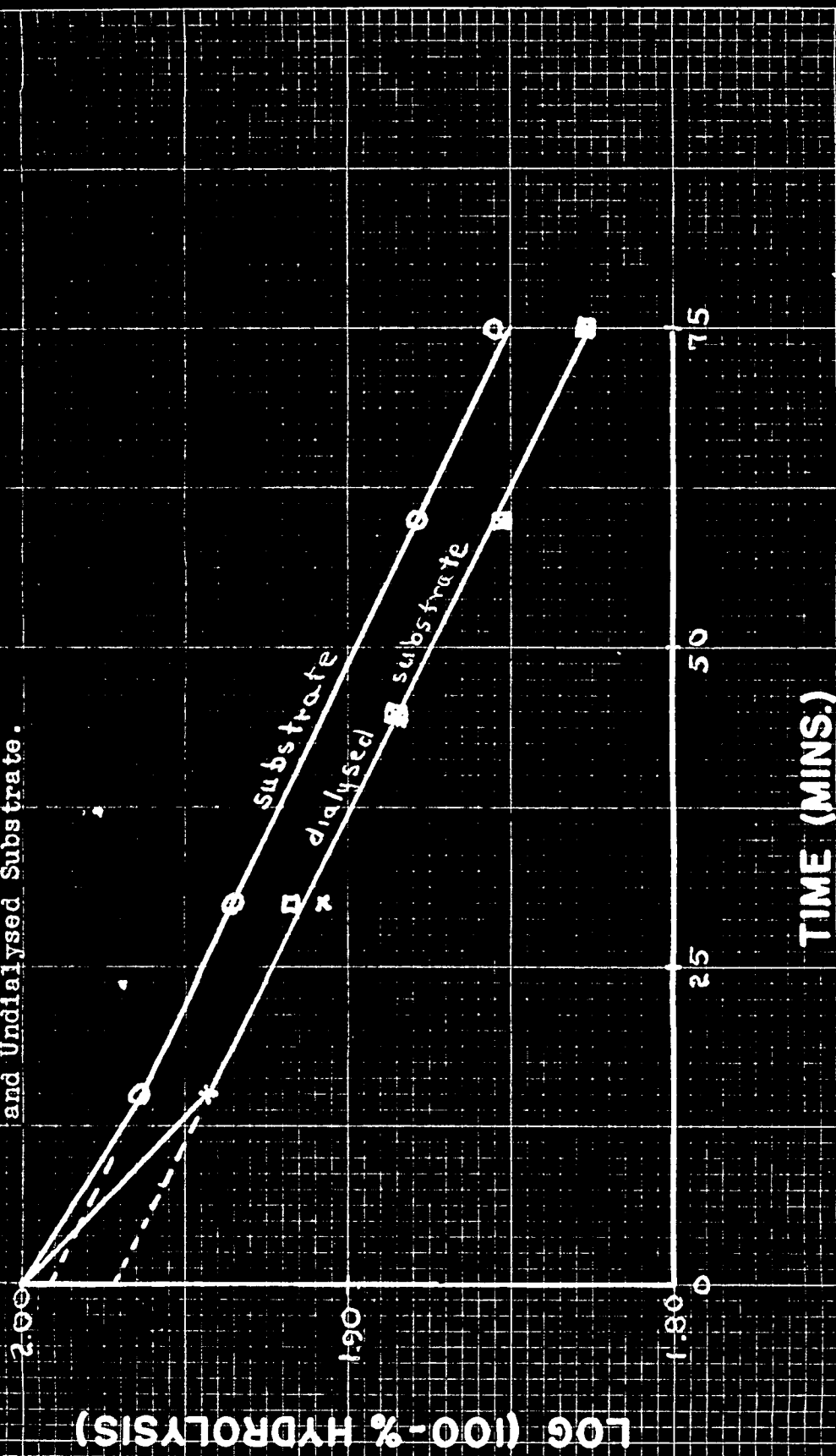


The rate of breakdown of A and B is

$$(1) \quad \frac{dA}{dt} = - xA \quad \text{and}$$

$$(2) \quad \frac{dB}{dt} = xA - yB.$$

Graph XI. The Determination of Rate Constants for Dialysed and Undialysed Substrate.



The formation of P_1 and P_2 is expressed by equations (3) and (4).

$$(3) \quad \frac{dP_1}{dt} = xA$$

$$(4) \quad \frac{dP_2}{dt} = yB$$

Integrating equation (1) gives

$$(5) \quad \ln A = -xt + c \quad \text{or}$$

$$(6) \quad A = c_1 e^{-xt}$$

(a) At $t = 0$, c_1 must equal the initial concentration of A, thus giving

$$(7) \quad A = A_0 e^{-xt}.$$

From equation (2)

$$(8) \quad dB = xA dt - yB dt \quad \text{or, from (7)}$$

$$(9) \quad dB = xA_0 e^{-xt} dt - yB dt.$$

$$(b) \quad \text{Let } B = uv. \quad \text{Then } dB = u dv + v du.$$

$$(c) \quad \text{Also, let } v du = -yB dt = -yuv dt.$$

Expression (9) becomes

$$(10) \quad u dv + v du = xA_0 e^{-xt} dt - yuv dt.$$

This now can be separated into two equations.

$$(11) \quad u dv = xA_0 e^{-xt} dt \quad \text{and}$$

$$(12) \quad v du = -yuv dt.$$

From equation (12)

$$(13) \quad \frac{du}{u} = -y dt \quad \text{or, on integration (e.g. (6) to (7))},$$

$$(14) \quad u = u_0 e^{-yt}$$

From equation (10) we derive that

$$(15) u_0 e^{-yt} dv - u_0 y v e^{-yt} dt = x A_0 e^{-xt} dt - u_0 y v e^{-yt} dt.$$

$$(16) u_0 e^{-yt} dv = x A_0 e^{-xt} dt \quad \text{or}$$

$$(17) dv = \frac{x A_0}{u_0} e^{(y-x)t} dt.$$

$$(18) v = \frac{x A_0 e^{(y-x)t}}{u_0 (y-x)} + c = \frac{B}{u_0} e^{yt} \quad \text{from (14).}$$

$$(d) \text{ Let } c = \frac{C_1}{u_0}. \quad \text{At } t = 0, B = 0, A = A_0 \text{ and (18)}$$

becomes

$$(e) \frac{x A_0}{(y-x)} + c_1 = 0 \quad \text{or}$$

$$(f) c_1 = - \frac{x A_0}{(y-x)}$$

Thus, equation (18) becomes

$$(19) \frac{x A_0 e^{(y-x)t}}{(y-x)} - \frac{x A_0}{(y-x)} = B e^{yt} \quad \text{or}$$

$$(20) \frac{x A_0 (e^{(y-x)t} - 1)}{(y-x)} = B e^{yt} \quad \text{and}$$

$$(21) B = \frac{x A_0}{(y-x)} (e^{-xt} - e^{-yt}).$$

In the formal titration, the sum of the concentrations of

$A + B + P_2$ is a constant equal to A_0 . Therefore, $P_2 = A_0 - A - B$.

From (7) and (21)

$$(22) P_2 = A_0 - A_0 e^{-xt} - \frac{x A_0}{(y-x)} (e^{-xt} - e^{-yt}).$$

$$(23) P_2 = \frac{A_0}{(y - x)} (y - x - ye^{-xt} + xe^{-yt}).$$

Again, because of the nature of the formol titration $P_1 = A_0 - A$ which gives

$$(24) P_1 = A_0 (1 - e^{-xt}).$$

Now, to get total product

$$(25) P_1 + P_2 = A_0 (1 - e^{-xt}) + \frac{A_0}{(y - x)} (y - x - ye^{-xt} + xe^{-yt})$$

or

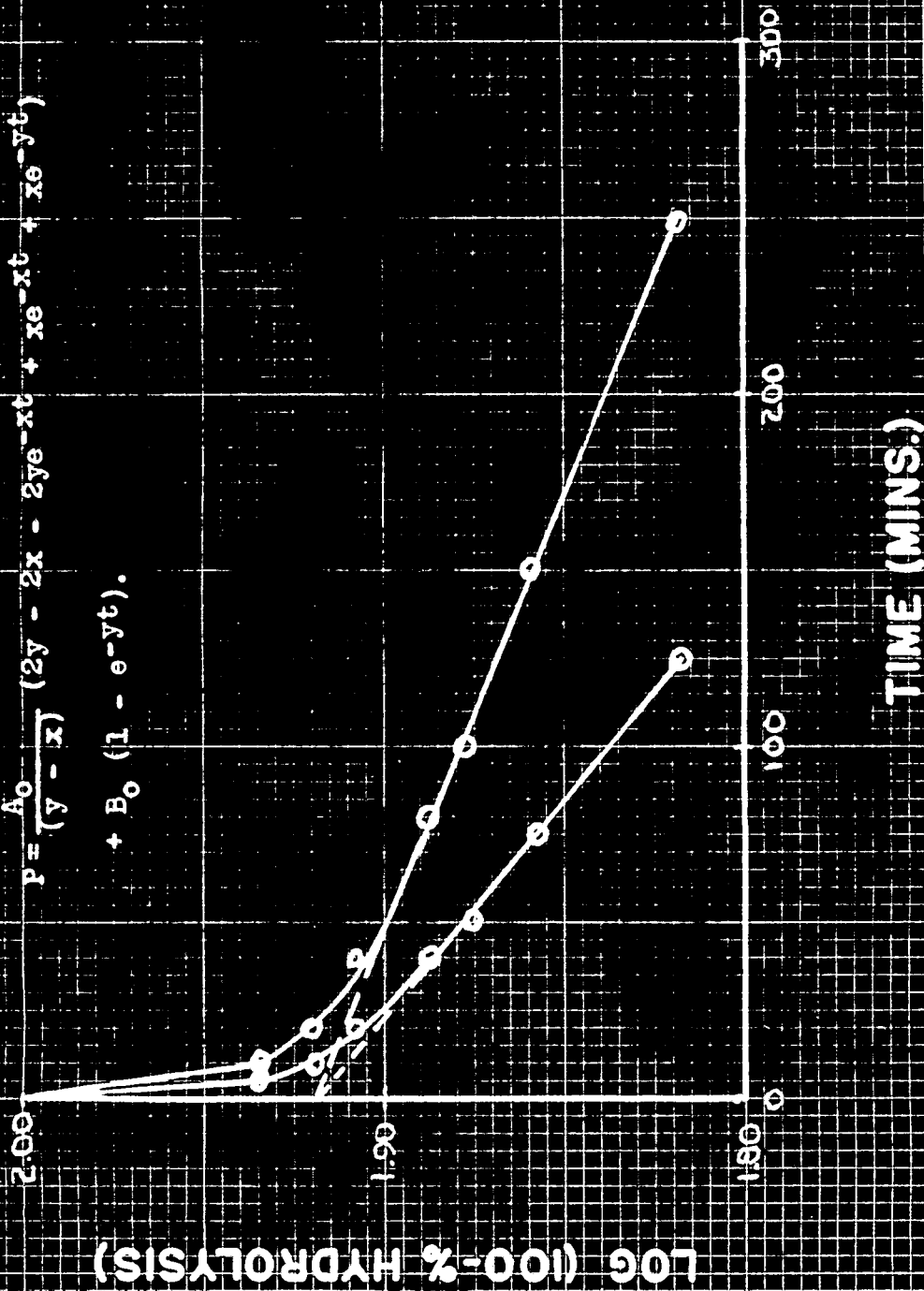
$$(26) P_1 + P_2 = \frac{A_0}{(y - x)} (2y - 2x - 2ye^{-xt} + xe^{-xt} + xe^{-yt}).$$

If instead of the formation of $1P_1$ or $1P_2$, we form instead $nP_1 + mP_2$, equation (25) is modified by a factor of n times the first expression and m times the second. Also, if the reaction starts with some amount of B present which can be called B_0 , then to (25) and (26) must be added $B_0(1 - e^{-yt})$, which is multiplied by m if more than one P_2 is formed.

A plot of (26) + $B_0(1 - e^{-yt})$ is given in Graph XXI, where $x = 0.1$, $y = 0.001$ and $B_0 = 10A_0$. The second plot shows what happens if the x and y values are doubled by doubling the enzyme concentration. It is evident that the apparent initial concentrations of substrate are less if the first order reaction is extrapolated back to zero time. However, the apparent initial concentration is independent of enzyme concentration which is not the case experimentally. In order to explain this discrepancy, one can postulate that the reaction $A \rightarrow B$ is inhibited by the reaction products. To test

Graph XXI. A Plot of the Equation:

$$P = \frac{A_0}{(y-x)} (2y - 2x - 2ye^{-xt} + xe^{-xt} + xe^{-yt}) + B_0 (1 - e^{-yt}).$$



this postulate an experiment was performed in which varying concentrations of end products from a previous hydrolysis were introduced into the reaction at zero time. All titration values were corrected for the titration values of the reaction products introduced. The results (Graph XXII) show that as the amount of reaction products increases, the apparent initial concentration of substrate increases. This is what would be expected if the reaction $A \longrightarrow B$ were inhibited by reaction products. It is also apparent that there is no appreciable inhibition of the second reaction, under the conditions given, since there is no change in slopes up to an amount of end products equivalent to 12.4% hydrolysis.

C. Effect of Temperature on Reaction Rate

In order to determine the molar energy of activation of the substrate before hydrolysis can occur in the presence of enzyme, the next experiments were performed. Velocity constants were determined for the isolated heat-labile and heat-stable enzymes at 30° and 40° C. The experiment was repeated with a different concentration of the enzymes at 35° and 45° C. The rate constants obtained are given in Table XIX. It should be noted that it was necessary to determine the initial rate constant for the heat-labile enzyme by extrapolating a plot of rate constants of individual samples vs. time back to zero time. This was necessary as there was some inactivation of enzyme with time. The molar energies of activation, "E," given in Table XIX, were obtained by substituting the rate

Graph XXII. The Effect of Varying the Concentration of End Products.

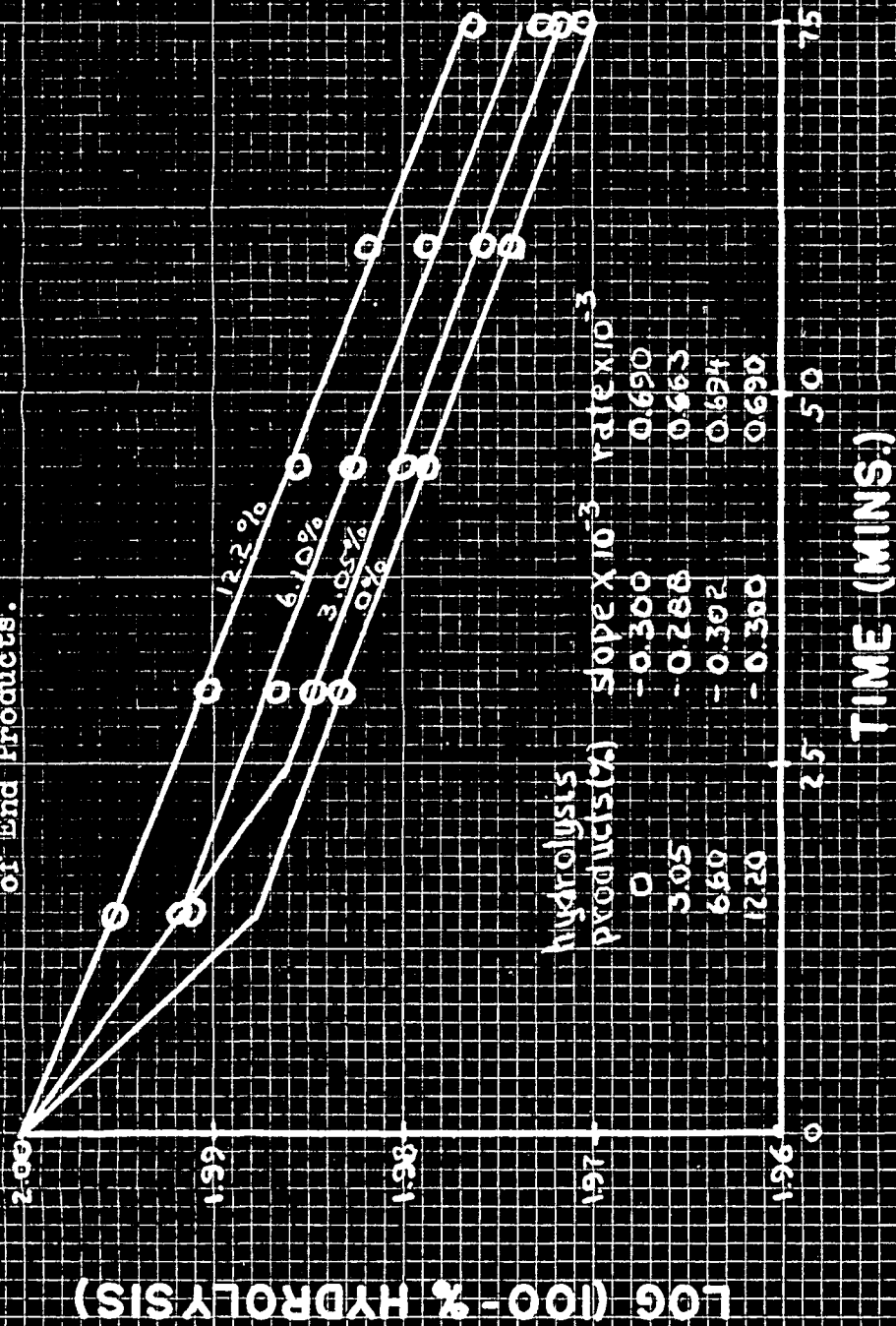


TABLE XIX

The Determination of Q_{10} and Molar Energy of Activation
for Heat-labile and Heat-stable Enzymes

Enzyme	Temp.	Rate Const. $\times 10^{-4}$	Q_{10}	$E \times 10^4$ (cal./mole)
Labile	30	0.9		
	40	1.6	1.8	1.08
	35	2.1		
	45	3.7	1.8	1.11
Stable	30	2.93		
	40	5.15	1.76	1.07
	35	2.90		
	45	5.33	1.84	1.16

constants found into the definite integral of the Arrhenius equation. The Q_{10} values given are somewhat lower than would be expected.

D. Reproducibility of rate measurements

Five samples of enzyme plus substrate were made up in the same manner and the rates were determined. The results, given in Graph XXIII, show that the reproducibility of this method is good and, therefore, differences obtained in comparative studies have a high degree of reliability.

V IMPROVEMENT IN ENZYME PRODUCTION IN CULTURES OF PROTEUS VULGARIS

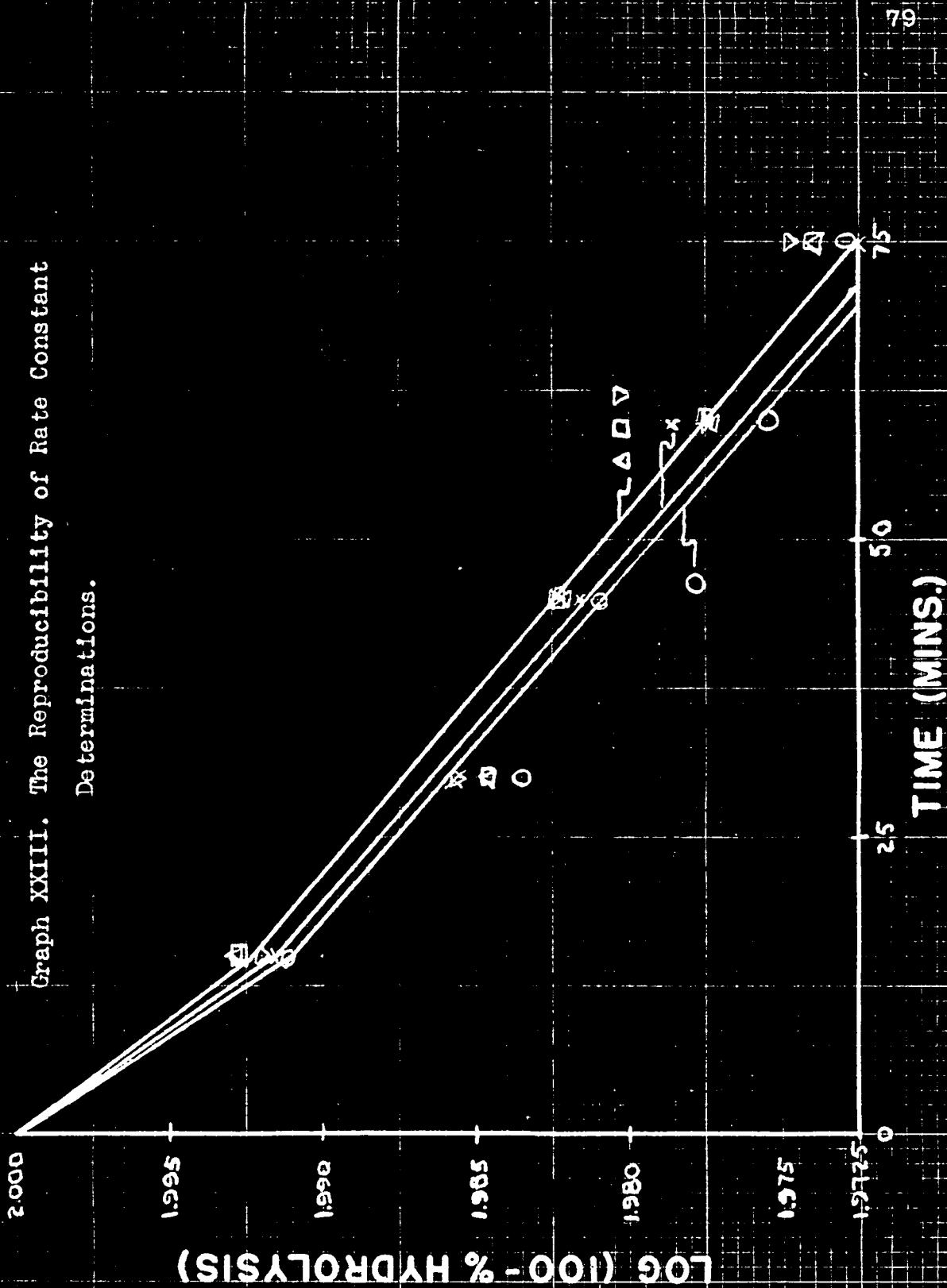
Although not of primary importance in this work, it was thought that some attempt should be made to improve the production of enzyme liberated into the culture medium by the bacteria.

A. The effect of calcium in the media

The culture medium used throughout this work was made by adding 15.0 gms. of Difco casamino acids and 5.0 gms. of Difco yeast extract to each liter of distilled water. The medium was adjusted to pH 7.8 - 8.0, dispensed in 1 - 1.5 liter quantities in 3-liter Fernbach flasks and sterilized in the autoclave for 20 minutes at 15 lbs./in.² pressure.

Many authors (13) (14) (15) (33) have claimed that calcium increases the yield of the proteolytic enzyme from

Graph XXIII. The Reproducibility of Rate Constant Determinations.



P. vulgaris without a concurrent increase in the number of bacteria. Gorini (11) demonstrated that the calcium was a means of stabilizing the enzyme.

Because of the above information, an experiment was performed to determine whether added calcium improved the quantity of active enzyme. To 100 ml. of medium in a 250 ml. flask was added 1 ml. of a 0.4% solution of CaCl_2 . An aliquot without added calcium served as a control. The culture media were inoculated with P. vulgaris and incubated 48 hours at 37°C . The cultures were filtered to remove the bacteria and the filtrate tested for enzyme using the film-strip method. Table XX lists the results.

Because of the fact that added calcium decreased the yield of enzyme, it was considered necessary to determine whether or not the medium had enough calcium in it without further addition. One gm. of casamino acids plus 0.33 gms. of yeast extract were fused using a NaNO_3 fusion mixture. The ash was dissolved in distilled water and analysed for calcium by the oxalate-permanganate method. It was found that each liter of medium contained 16 ± 2 mg. of calcium, which was 1/10 the amount used by Gorini. Since the amount which was added to the flask in the first experiment was the same as used by Gorini, it was concluded that 16 mg. Ca/liter was closer to the optimum amount.

TABLE XX

The Effect of Calcium on Enzyme Activity

Calcium conc.	0.004%		None	
start	65	65	60	60
finish	80	80	65	65

B. Optimum pH of the medium and optimum
time of incubation

Seven 100 ml. aliquots of culture medium were adjusted to a series of pH values before sterilization. Each was inoculated with 1.0 ml. of a 48 hour culture of P. vulgaris, and incubated at 37° C. At varying intervals of time, 9 ml. samples were removed and the pH recorded. The samples were sterilized by the addition of 1 ml. of a saturated solution of phenol in water. The enzyme solutions were then dialysed against running water for 18 hours and assayed by the second method of the formol titration after 3 1/4 hours incubation (Table XXI).

Although the results are not clear cut, they indicate that the optimum time of incubation is from 70 to 96 hours at an initial pH of 7.5 - 8.1. It is interesting to note that the pH climbs steadily with time until it reaches the same maximum in all flasks after 96 hours.

The above results are in accordance with the findings reported by Merrill and Clark (27), who stated that the optimum initial pH for the production of the enzyme was pH 7 - 8. Derby (5), however, found it to be pH 6 - 7.

The fact that the largest percentage of enzyme production occurs during the time of maximum growth indicates that the enzyme is secreted by the bacteria. Additional enzyme is liberated during the period of autolysis. These observations, using P. vulgaris, confirm those of many authors working with other bacteria (3) (9) (10) (4).

TABLE XXI

Optimum Time and pH of Incubation on Production of Enzyme

Initial pH	Time (hrs.)	microeq. NaOH used	Final pH	Time (hrs.)	microeq. NaOH used	Final pH
6.3	25.5	3.16	7.28	45	3.47	7.90
6.6	25.5	3.81	7.35	45	3.90	7.97
6.9	25.5	2.92	7.79	45	3.59	8.18
7.2	25.5	3.06	7.58	45	3.25	8.03
7.5	25.5	(4.43)	8.00	45	3.95	8.29
7.8	25.5	4.02	7.95	45	4.05	8.23
8.1	25.5	4.00	8.18	45	4.10	8.37
6.3	70.5	3.78	8.23	96	---	8.69
6.6	70.5	3.66	8.23	96	3.32	8.70
6.9	70.5	3.90	8.27	96	3.78	8.70
7.2	70.5	3.23	8.26	96	3.61	8.67
7.5	70.5	4.14	8.43	96	4.32	8.80
7.8	70.5	4.36	8.32	96	3.85	8.77
8.1	70.5	4.07	8.47	96	4.40	8.79

C. Comparison between production at room temperature and at 37° C.

Two 100 ml. aliquots of medium, adjusted to pH 7.8, were inoculated with 1 ml. of a 24-hour culture. One flask was incubated at 37° C. while the other was placed out in the laboratory. Nine ml. samples were removed at the stated times, phenolized and assayed as in the preceding experiment.

The results in Table XXII indicate that the yield of enzyme is greater when incubation is carried out at room temperature. This is in agreement with the findings of Gorini (11).

VI. THE LARGE-SCALE PRODUCTION OF ENZYME

After many large-scale lots of enzyme had been produced, the method about to be described was evolved. Twelve liters of culture media containing 15 gms. of casamino acids and 5 gms. of yeast extract per liter of solution at pH 7.8 were dispensed in 8, 3 l. Fernbach flasks and sterilized in the autoclave. The medium in each flask was inoculated with approximately 5 ml. of a 24-hour culture of P. vulgaris. The cultures were incubated 4 days at room temperature. A filter was prepared by packing a 1" layer of Johns-Manville high-flow filter cell on top of a 1/2" layer of Johns-Manville Celite in a 10" Buchner funnel, equipped with a 2 l. suction flask. The bacterial cultures were filtered. The filtrate was adjusted to pH 5.5, 120 gms. of kaolin were added, and

TABLE XXII

The Effect of Temperature on Production of Enzyme

Temperature	Time	microeq. NaOH used
Room	24	2.05
Room	72	3.61
37° C.	24	1.75
37° C.	72	2.80

the mixture stirred for 20 minutes. The kaolin was allowed to settle and the supernatant liquid was siphoned off. The kaolin was then isolated by filtration through a 10" Buchner funnel and washed with 4, 125 ml. portions of 0.04 M acetate buffer, pH 5.5. The enzyme was eluted by passing 1 l. of a 0.15% ammonia water solution through the kaolin. The eluent was dialysed against cold tap water for 3 days. The volume of the eluent was reduced to about 200 ml. by pervaporation and then frozen. After thawing out, the solution was filtered to remove the precipitate. The enzyme was then obtained as a dry powder by lyophilization.

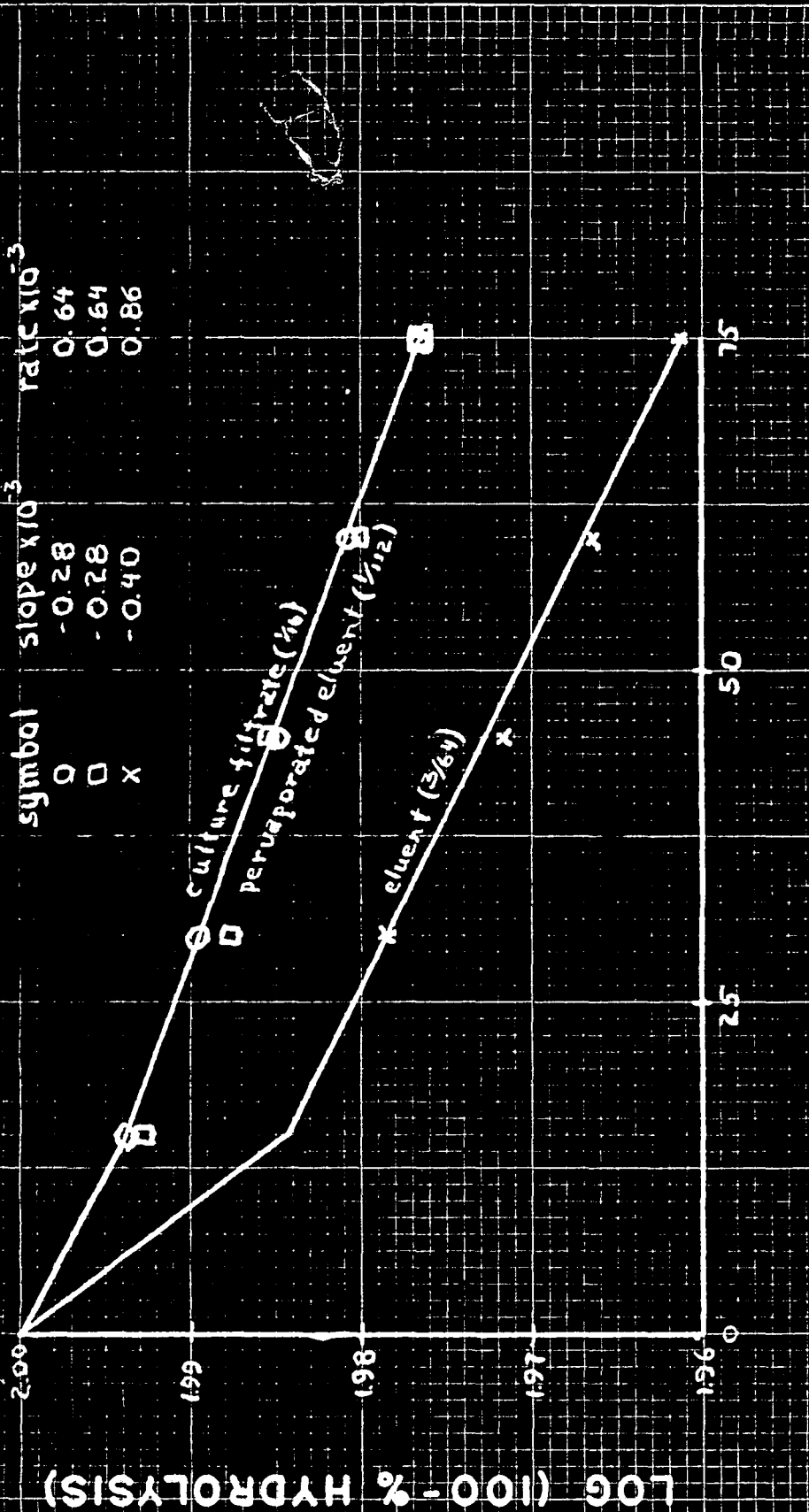
Graph XXIV gives the rate constants of the enzyme at various steps in the production, as obtained by the second method of formol titration. Using the following facts, we can calculate the yield:

volume of dialysed culture filtrate:	12,000 ml.
volume of eluent:	1,000 ml.
volume of pervaporated eluent:	106 ml.
dilution of dialysed culture filtrate assayed:	1/16
dilution of eluent assayed:	3/64
dilution of pervaporated eluent assayed:	1/112
rate constant of dialysed culture filtrate:	0.00064
rate constant of eluent:	0.00086
rate constant of pervaporated eluent:	0.00064

The percentage yield of eluent is $86/64 \times 64/3 \times 1/16 \times 1000/12,000 \times 100$ or 15%. The overall yield is $34/34 \times 112/1 \times 1/16 \times 106/12,000 \times 100$ or 6%. A loss of $(20-6)/20$ or 60% occurred during pervaporation. If 1 unit of enzyme is considered to have a rate constant of 0.0001, and since the yield of dry powder was 300 mg., we can calculate an activity

Graph XXIV. Assay of Enzyme at Different Steps in the Purification.

symbol	slope $\times 10^{-3}$	rate $\times 10^{-3}$
○	-0.28	0.64
□	-0.28	0.64
x	-0.40	0.86



TIME (MINS.)

of $\frac{112 \times 106 \times 6.4}{300}$ or 250 units per mg. of dried powder,

assuming that there was no loss of activity during the lyophilization.

VII. SUMMARY AND CONCLUSIONS

1. The optimum conditions for the production of proteolytic enzymes in cultures of P. vulgaris are an initial pH of 7.5 - 8.1 and a 3 - 4 day incubation period at room temperature when a casamino acids-yeast extract medium is used.
2. The method of purification found to be most suitable is adsorption of the enzyme from the culture filtrate on 1 gm. of kaolin per liter of solution at pH 5.5, washing the adsorbed enzyme with an acetate buffer at pH 5.5, and elution using a dilute ammonia water solution. The dry enzyme obtained from the eluent contains at least three proteins, two of which are proteolytic enzymes. The boundaries formed by the two enzymes on electrophoresis were identified. The more rapid-moving component at pH 5.5 and $\eta/2$ of 0.1 is a relatively heat-stable enzyme. The other active component is a heat-labile enzyme.
3. The results from the study of the rate of inactivation of the enzyme preparation at temperatures of 60 - 100° C. again indicate the presence of two enzymes.

4. By determining the velocity constants of the two enzymes with varying concentration of substrate and substituting these values in the Michaelis-Menten equation, a value of approximately 50 is obtained for the Michaelis-Menten constant.
5. It was found that the heat-stable enzyme is partially inhibited by the gelatin substrate.
6. A unit of enzyme based on rate-constant determinations is suggested.
7. It was observed that the apparent initial concentration of substrate appeared to be less than the actual amount when the first order plot of rate constant was extrapolated to zero time. To explain this discrepancy, it is postulated that there is an initial rapid breakdown of larger molecules and a slower breakdown of these products to still smaller fragments. The kinetics are expressed mathematically in an equation, which, when plotted using assumed values for rate constants, appears to be similar to plots from experimental data.
8. Molar energies of activation are found to be 1.1×10^4 cal. for both enzymes.

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ABSTRACT

1. Methods of Assay

Two variations of the formol titration were developed to determine proteolytic activity. The first used phenolphthalein as an end-point indicator while the second was a potentiometric titration with an end point of pH 7.8. The standard error of the mean for the two methods were ± 0.3 and ± 0.13 microeqs. respectively. The substrate used was a 3% gelatin solution with a tris (hydroxymethyl) aminomethane buffer at pH 7.4, the optimum pH of enzyme activity.

2. Production of Enzyme in Bacterial Cultures

The culture medium used contained 15 g of Difco casamino acids and 5 g of Difco yeast extract per liter of distilled water. The medium was adjusted to the proper pH, dispensed in 1.5-liter amounts into 3-liter Fernbach flasks. The medium was then sterilized by heating for 20 minutes at 120 C. Ten ml of a 24-hour culture of P. vulgaris was used as the inoculum.

The results of experiments showed that the optimum conditions for the production of the enzyme were an initial pH of the medium of from 7.5 to 8.1 and a 4-day incubation period at room temperature.

3. Purification

The purification of the enzyme was attempted by precipitation using organic solvents. The optimum conditions

for precipitation were a 30% (v/v) alcohol concentration at pH 4.5 and an ionic strength of 0.016 or a 5% (v/v) acetone concentration at pH 5.0. The yields were too poor to be of use.

The method of purification which was finally evolved used kaolin as an adsorbing agent. Cultures of P. vulgaris were filtered through Celite. The pH of the filtrate was adjusted to 5.5 and 10 g of kaolin were added to each liter of filtrate. After mixing for from 10 to 20 minutes, the kaolin was allowed to settle and the supernatant liquid was discarded. The kaolin was washed on a filter using 4 or 5 portions of a 0.04 M acetate buffer at pH 5.5. The enzyme was eluted using a 0.15% ammonia-water solution to one tenth the volume of the culture filtrate. The mixture was filtered and the kaolin discarded. The enzyme solution was dialysed against tap water. The enzyme could then be isolated as a dry powder by pervaporation followed by lyophilization. A 15% - 20% recovery of enzyme was obtained after elution and a 6% over-all recovery was obtained with a 5-fold purification as compared to a sample of a dialysed culture filtrate.

Electrophoretic data showed that there were three main components in the isolated, dried enzyme. One component with a mobility of -12.1×10^{-5} cm²/volt-sec for the ascending limb at pH 5.5 and an ionic strength of 0.1, was inactive while two components, with mobilities of -1.23 and -3.70 cm²/volt-sec

under the same conditions, were active. The more rapid active component was found to be a heat-labile enzyme, while the slower was relatively heat stable.

4. Properties of the Enzymes

Casein, autoclaved tendon, gelatin, and fibrinogen were hydrolysed by the enzymes while raw tendon, Ribonucleic acid and Desoxyribonucleic acid were not.

Cysteine, Fe^{++} , and cysteine + Fe^{++} were found to inhibit the enzymes. Mg^{++} , Sn^{++} , and Cu^{++} inhibited the enzymes, Ca^{++} , Ba^{++} , Mn^{++} , and Zn^{++} had no effect.

5. Enzyme Kinetics

The first step necessary for the study of the kinetics of the enzyme system was to determine the per cent hydrolysis represented by each microeq. of NaOH used in the titration and the concentration of gelatin, as microeq. of peptide bonds. To determine these facts, various concentrations of gelatin were incubated 18 hours with a concentrated preparation of enzyme. The amount of hydrolysis was then determined by the biuret method. A series of tubes was made up representing a series of percentages of hydrolysis and titrated. It was found that there were 20 microeqs. (20 microeq. NaOH) of peptide bonds per % substrate.

The results of the determination of the thermal inactivation rate of the enzyme demonstrated that there were, in fact,

two proteolytic enzymes, a heat-stable and a heat-labile enzyme, a fact also demonstrated by the results of the electrophoretic study.

In a determination of per cent hydrolysis at increasing periods of time, it was found that the hydrolysis proceeded according to a first order rate and that the rate constant was proportional to enzyme concentration. Therefore, a unit of enzyme was proposed making one unit equivalent to a rate constant of 0.0001 using a 3% concentration of gelatin substrate when incubated at 37 C.

The rate constants were determined for substrate concentrations of 1 - 6% and varying enzyme concentrations. It was found that the rate constants for equivalent enzyme concentrations decreased as the substrate concentration increased. Thus, it may be seen that there was substrate inhibition of the enzyme preparation.

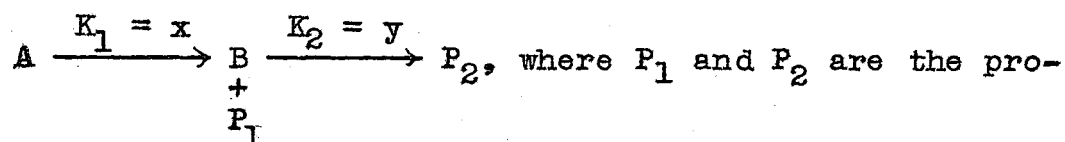
The determinations of rate constants with varying substrate and enzyme concentrations were repeated for the heat-stable enzyme obtained by heating the mixture at 100 C. for 2 minutes. Again, there was noticeable substrate inhibition of enzyme activity.

In order to determine the activity of the heat-labile enzyme, it was necessary to determine the amount of heat-stable enzyme left after heating the enzyme mixture. By heating the mixture to destroy the heat-labile enzyme, a sample of heat-stable enzyme was obtained. Fifty-six per cent of the enzyme

was found to remain after heating the sample of heat-stable enzyme at 100 C. for 2 minutes. By dividing the rate constants of the heat-stable enzyme by 0.56, the values for the heat-stable enzyme in the mixture, before heating, were obtained.

In order to substitute the values into the Michaelis-Menten equation, it was necessary to convert rate constants into velocities. This was done for the values for the enzyme mixture and the heat-stable enzyme corrected for loss on heating. By subtracting the reaction velocity caused by the heat-stable enzyme from the reaction velocity of the enzyme mixture, for the same concentration of enzyme, the velocity of the heat-labile enzyme was obtained. By substituting the velocity and substrate values into the Michaelis-Menten equation, a value of 9×10^{-4} M was obtained for the Michaelis-Menten constant for each enzyme calculated separately.

It was noticed that the plots of the first order reaction did not extrapolate back to 0% hydrolysis at zero time but to some positive value. As the concentration of enzyme increased, the value of substrate at zero time increased. In other words, there was a fast initial reaction. The value of the intercept at zero time was greater when the gelatin was dialysed and the buffer replaced. This fact indicated that the initial reaction was the hydrolysis of the larger molecules. To explain these findings mathematically, the following simplified sequence of reactions was postulated:



ducts which are titrated after subtracting the titration value of the substrate. Thus, the expressions for the breakdown of A and B and production of P_1 and P_2 are

$$(1) \frac{dA}{dt} = -xA, \quad (2) \frac{dB}{dt} = xA - yB,$$

$$(3) \frac{dP_1}{dt} = xA, \quad \text{and} \quad (4) \frac{dP_2}{dt} = yB$$

By changing B, in equation (2), into values of A, integrating, converting these into expressions of rate of product formation and finally adding the equations for the formation of products, we obtained the following expression for the production of total products:

$$(5) P_t = \frac{A_0}{y-x} (2y - 2x - 2ye^{-xt} + xe^{-xt} + xe^{-yt}).$$

Since the substrate contains an initial amount of B, a value of $B_0(1 - e^{-yt})$ must be added to equation (5).

It will be seen from equation (5) that if x is greater than y , the values of e^{-xt} will become negligible as compared to e^{-yt} and the expression would reduce to a first order reaction after a short period of time. However, it was found by plotting the equation that the zero-time intercept was independent of enzyme concentration, which is contrary to experimental findings. Therefore, it was postulated that the initial reaction

was inhibited by reaction products, whereas the second reaction was not. This was actually found to be the case when reaction rates were determined for a series of reactions when end products, representing up to 12% hydrolysis, were introduced at zero time.

The reaction rates were determined at 30°, 35°, 40°, and 45° for the two enzymes isolated by electrophoresis. From these rates a Q_{10} of 1.8 and a molar energy of activation of 1.1×10^4 cal. were found for both enzymes.

6. Summary

The production and purification of the proteolytic enzymes of Proteus vulgaris are discussed. Kaolin was used as an adsorbent in the purification. Subsequent elution yielded a preparation containing two proteolytic enzymes and an inactive fraction. The active fraction consists of a relatively heat-stable enzyme and a heat-labile enzyme. The kinetics of these two enzymes was studied to determine the Michaelis-Menten constants, effects of substrate, effect of end products, the molar energy of activation, and the inactivation by heat. A unit of activity based on reaction rates is proposed.

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