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I hereby recommend that the thesis prepared under my supervision by James D. Ogle

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CLOSTRIDIUM HISTOLYTICUM COLLAGENASE AND PROTEINASE

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CLOSTRIDIUM HISTOLYTICUM COLLAGENASE AND PROTEINASE

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by

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PURPOSE OF THE INVESTIGATION

The objectives of this investigation were:

1) To determine whether *Clostridium histolyticum* collagenase and proteinase had any specificity toward synthetic substrates,
2) To develop simple and reliable methods for the detection and assay of one or both of these enzymes in crude enzyme preparations,
3) To attempt to purify the proteinase by altering the conditions of its precipitation from cell-free filtrates.

INTRODUCTION

It has been known for many years that proliferating cells of Clostridium histolyticum secrete proteolytic enzymes into the culture medium. Two of these extracellular enzymes have been studied considerably in recent years. These are "collagenase," which attacks native fibrous collagen and "proteinase," which hydrolyzes gelatin, casein, clupein, and other protein substrates but does not attack collagen. The importance of these extracellular enzymes of Cl. histolyticum lies in their association with the metabolism of Cl. welchii in the well known gas gangrene infection. It is believed that the extracellular proteinases of the non-pathogenic Cl. histolyticum and of other Clostridia hydrolyze the tissue proteins and thus make available peptides and amino acids for the metabolism of the less proteolytic but highly pathogenic Cl. welchii. Collagenase is unique in this scheme because it attacks the collagenous sarcolemma of the striated muscle and thus exposes the muscle tissue to complete proteolysis. It is not known whether collagenase itself further hydrolyzes the "degraded" collagen and the tissue protein once the collagen structure is destroyed or whether another proteinase accomplishes this. Nor is the manner in which collagenase attacks native collagen known.

Although collagen has been classified as a simple protein it has been reported that there is from 0.1% to 1% of carbohydrate in the molecule. The attack on collagen by collagenase may be at the site of this carbohydrate and thus collagenase may not actually be a proteolytic enzyme from the standpoint of hydrolyzing peptide bonds.

The "proteinase" of Cl. histolyticum, on the other hand, is truly a proteolytic enzyme. It has been reported by Maschmann (1), Kocholaty (2,3,4) and van Heyningen (5), and has been characterized by these investigators with respect to such protein substrates as gelatin, casein, and clupein. There is some disagreement in the results of these investigators. Maschmann reported that there was one or more extracellular proteinases secreted by Cl. histolyticum and that these proteinases were inhibited by cysteine. Maschmann maintained that only during later stages of growth of the cells was an intracellular proteinase elaborated (probably by autolysis) which had some "initial" activity without activation by cysteine but which was activated further by cysteine. Kocholaty reported only one proteinase, extracellular and activated by cysteine, although there was some "initial" activity without cysteine. van Heyningen described an extracellular proteinase, activated by cysteine but with no "initial" activity. By repeated subculturing

on a different medium van Heyningen was able to obtain an extracellular proteinase inhibited by cysteine, plus, later in growth and after autolysis, an intracellular proteinase activated by cysteine but still with "initial" activity without cysteine. In the cases where the proteinase was activatable by cysteine it was observed by all the investigators that the proteinase was further activated by ferrous sulfate plus cysteine. In some cases these same proteinases were inhibited by ferrous sulfate alone while in other instances they were activated by ferrous sulfate alone.

van Heyningen pointed out three important facts as a result of his work:

1) Activation behavior of the culture filtrates; that is, the actual nature of the proteinases secreted, varies with the strain of Cl. histolyticum.
2) Earlier disagreements in results were probably due to the presence of at least two proteinases, one cysteine activatable, the other inhibited by cysteine. Thus there would be no net change in activity as a result of adding cysteine to the proteinase mixture.
3) Estimation of proteinase activity of the enzyme mixtures by measuring release of free amino or carboxyl groups was invalid since poly- and di-

peptidases, known to be present in the mixtures, would also attack the partially hydrolyzed protein substrate. Also, any method involving protein substrates necessarily gave a measure of total proteinase activity.

The accurate characterization of an enzyme-protein substrate system is obviously limited by the lack of knowledge of the structure of the substrate and by the lack of purity of the enzyme preparation.

In view of the shortcomings of complicated protein substrates for characterizing enzyme systems, it seemed advantageous to use simple synthetic substrates for which the nature of the enzymatic reactions could be determined. Simple amino acid derivatives, amides, esters, and peptides have been used successfully for many enzymes. Therefore a series of amino acid esters, amides, and peptides was prepared to determine the synthetic substrate specificity of Cl. histolyticum collagenase and proteinase. Thus having established this specificity, methods for measuring enzymatic activity could be devised and the enzymes could be characterized with respect to pH optima, stability, and activation and inhibition behavior.

METHODS

Growth of Cells and Production of Enzymes

The strain of Clostridium histolyticum used in this study was CHT from the stock culture collection of this laboratory. Stock cultures were maintained on proteose-peptone (Difco) over beef heart as described below.

Preparation of Dry, Solvent Extracted Beef Heart. Fresh beef heart was carefully freed of gross fat and was finely ground in a meat chopper. One kg. of ground tissue was kneaded with 3 liters of 95% ethanol for 4 hours. The alcohol was removed by decantation and straining through cheese cloth. The alcohol extracted tissue was re-extracted with 3 liters of 1:1 ethanol-ether with stirring for 4-8 hours. The solvent was removed and the tissue re-extracted twice more with two-liter quantities of ether. After decantation of the ether the tissue was spread in a thin layer and dried at room temperature in a current of air. The dry, fat-free beef heart was light tan in color. It could be stored in a covered jar at room temperature for several weeks.

Preparation of Stock Medium. Twenty grams of proteose-peptone (Difco) were dissolved in one liter of distilled water with warming. The solution was adjusted to pH 7.4 to 7.5 with NaOH. Forty-ml. quantities were transferred to 25 x 200 mm. pyrex culture tubes, containing one

gram of solvent extracted beef heart per tube. The tubes were plugged, then autoclaved at 15 pounds for 30 minutes. If the stock medium was to be used immediately, the tubes were cooled rapidly to room temperature prior to inoculation. Otherwise the stock medium tubes could be stored at room temperature for two weeks.

Sodium Thioglycolate Solution. 0.25 ml. (3.6 millimoles) of thioglycolic acid was pipetted into a 25 x 200 mm. pyrex tube, 9 ml. of distilled water and 0.75 ml. of 5 N NaOH were added. The solution was faintly alkaline to phenolphthalein. The tube was plugged, then autoclaved at 15 pounds for 10 minutes. The solution was cooled to room temperature before use in stock culture tubes. This solution was made up fresh each day it was to be used.

Stock Cultures. When the stock medium tubes had been stored after autoclaving, they were heated prior to inoculation in a boiling water bath for 20 minutes to expel air. After cooling to room temperature one ml. of the sterile sodium thioglycolate solution was added to each tube under aseptic conditions. Each tube was then immediately inoculated aseptically with 2 ml. of the previous stock culture. They were then incubated at 37° C. for 18 hours, then stored in the cold room (4° C.). The cultures were transferred every two weeks. Two cultures were carried, transfers

being made from each tube. Slides were made periodically to check for contamination.

Tryptic Digest of Casein. 1100 grams of casein were suspended in 8 liters of distilled water and stirred vigorously. The suspension was heated to 50° C. and adjusted to pH 8.3 with NaOH. The NaOH was added very slowly and the suspension was stirred very vigorously after each addition. 45 grams of trypsin (1:100) were suspended in enough distilled water to make a smooth slurry. This slurry was added to the casein suspension, covered over with 600 ml. of toluene and incubated at 45° C. for 24 hours. After incubation the toluene was removed by suction, the digest was adjusted to pH 5 with HCl and boiled for 20 minutes. The digest was then filtered hot through two layers of filter paper with a small amount of filter-cel in the cone of the funnel. The solid content of the clear digest was determined; it was usually between 12% to 13%. The digest was stored under toluene in the cold room.

Growth Medium for the Production of Enzymes. The amount of tryptic digest of casein required to give 6 liters of 4.5% solids was calculated. Slightly more than this amount was taken to allow for loss in subsequent filtration, and the toluene was removed. 34.6 grams of $Na_2HPO_4 \cdot 12H_2O$ and 2.88 grams of KH_2PO_4 were dissolved in a small amount of distilled water and boiled several minutes, then added to the

digest. The solution was adjusted to pH 7.2 to 7.4 with NaOH (phenol red), boiled 10 minutes and filtered hot through two layers of filter paper with Super-cel in the cone of the funnel. The filtrate was cooled, adjusted accurately to pH 7.2 to 7.4, and an amount necessary to give 3 liters of 4.5% solids was poured into each of two 4-liter bottles. Each bottle was diluted to 3 liters with distilled water, plugged, and autoclaved at 15 pounds for 40 minutes. The bottles were cooled to around 37° C. before inoculation. A sodium thioglycolate solution for each growth medium bottle was prepared as follows: 1.9 ml. of thioglycolic acid were pipetted into each of two 25 x 200 mm. pyrex tubes. Thirty ml. of distilled water and 5.6 ml. of 5 N NaOH were added to each tube. The tubes were plugged, autoclaved at 15 pounds for 10 minutes, cooled to room temperature, and added aseptically to the growth medium bottles. Next, each bottle was inoculated aseptically with 40 ml. of fresh culture and incubated at 37° C. for 18 hours. The cultures were transferred and grown out for two consecutive 18 hour periods preceding inoculation of the growth medium bottles.

Filtration-Preparation of the Cell-Free Filtrate. After incubation, the contents of the growth medium bottles were filtered under slightly reduced pressure through Super-cel in a 25 cm. Büchner funnel. The Büchner funnel was prepared as follows: two layers of filter paper, moistened,

were fitted tightly on the bottom of the funnel with suction. A thin suspension of Super-cel was poured over the filter paper and just before all the water had filtered through, a filter paper was placed over the Super-cel and a small amount of water was poured over the filter paper. The Super-cel cake was about 1 cm. thick. Just before all of the water had filtered through, some of the growth medium was poured into the funnel and suction applied. When the filtrate came through clear and turned red (ferric-thioglycolate complex), the receiving flask was changed and the remainder of the 6 liters of cell suspension was filtered. The clear, reddish-brown filtrate was then filtered through a 12 inch Berkefeld "N" candle. The filtrate obtained was the cell-free filtrate from which the collagenase and proteinase preparations were made. The pH of this filtrate was always around 7. The cell-free filtrate could be stored at 4° C. for as long as two months without destruction of the enzymes.

Preparation of Collagenase. The collagenase preparations used for kinetic studies were supplied by K. Hewson and were prepared essentially as follows (6): The cell-free filtrates were poured into Visking casing (1-5/8") and dialyzed against cooled running tap water in a continuous rocking dialyzer for 18 hours. To each 1500 ml. of dialyzed filtrate at 0° C., 1000 ml. of precooled methanol were added with constant stirring. The precipitate was allowed to form

and flocculate for 24 hours during which time the precipitate settled to the bottom of the container. The supernatant fluid was carefully siphoned off and the precipitate collected in a refrigerated centrifuge. The methanol precipitate from 1500 ml. of dialyzed filtrate was taken up in 40 ml. of water, and 20 ml. of 0.1 M phosphate buffer at pH 7.2 was added. The solution was brought to 75% saturation with ammonium sulfate at room temperature. The precipitate was allowed to form at room temperature for 24 hours, then collected in a refrigerated centrifuge. This precipitate was then dissolved in 20 ml. of water and centrifuged to remove insoluble material. The clear supernatant was used as such or was dried by lyophilization. Later, other collagenase preparations were made in essentially the same way except that they were fractionated from 40% to 75% saturation with ammonium sulfate.

Preparation of Proteinase. The proteinase preparations used for kinetic studies were prepared according to the following procedure: To each liter of cell-free filtrate (pH around 7) at -5° C., two liters of pre-cooled methanol were added with constant stirring. The precipitate was allowed to form and settle to the bottom of the container. The supernatant fluid was siphoned off and the precipitate collected in a refrigerated centrifuge. The precipitate was extracted with 25 ml. of 0.1 M phosphate buffer, pH 7.2, or

with 25 ml. of water when the effect of different buffers or of heavy metal salts was to be determined. After centrifugation the clear supernatant was stored at 4° C. for 24 hours, during which time a slight precipitate formed. This precipitate was centrifuged off and discarded and the clear supernatant could then be stored at 4° C. for months without further precipitation or appreciable loss of activity. The enzyme solutions thus prepared varied from colorless to pale yellow in color.

Several modifications in the method of preparation of the proteinase were tried in an attempt to purify the enzyme preparation. These modifications included varying the pH of the cell-free filtrate before precipitation; varying the amount of methanol used to precipitate the enzyme; precipitation with acetone instead of methanol; and precipitation with ethanol in the presence of zinc acetate. These modifications and the results are described on page 84.

Determination of Nitrogen Content of Enzyme Preparations. The nitrogen content of enzyme preparations was determined by the micro-Kjeldahl method. Determinations were run in duplicate. A 1:10 dilution of the enzyme preparation was made. Six-tenths ml. of the 1:10 dilution was pipetted into a 25 x 200 mm. pyrex tube, 0.2 ml. of digestion mixture, and a few particles of carborundum were added. The

contents of the tube were heated rapidly until all the water was dispelled and the tube was filled with SO_3 fumes. The flame was removed and the tube was allowed to cool for one minute. One drop of 30% H_2O_2 (Superoxol) was added and the tube was covered with an inverted 50 ml. beaker. The flame was replaced and the contents were heated for 10 minutes at such a rate that condensation of the SO_3 fumes occurred half way up the tube. The tube was then allowed to cool for several minutes, 10 ml. of distilled water were added followed by 4 ml. of Nessler's reagent, and 5 ml. of 2 N NaOH in that order. A blank was run in which water replaced the diluted enzyme solution. The tubes were read against the blank in an Evelyn photoelectric colorimeter with filter No. 515 and at the 10 ml. level. The nitrogen was determined from a standard curve in which optical density (= 2-log %T) was plotted against micrograms of nitrogen, see Figure 1. The nitrogen content was expressed as mg. nitrogen per ml. of enzyme preparation.

Digestion Mixture:-

Five grams of dry K_2SO_4 (Analytical Grade, low nitrogen content) was dissolved in 10 ml. of concentrated H_2SO_4 with exclusion of air.

Nessler's Reagent:-

Four grams of KI and 5.5 grams of HgI_2 were dissolved in 25 ml. of water, and added to a solution of 1.75 grams of gum ghatti in 750 ml. of water (dissolved by sprinkling powdered gum ghatti into the boiling water). The solution was diluted to 1 liter and filtered.

Methods of Determining Enzymatic Activity

Determination of "Collagenase" Activity toward Collagen. The activity of enzyme preparations toward collagen was determined by the method of Tytell and Hewson (6). In this method activity is measured by the release of soluble nitrogen from collagen. The desired amount of enzyme preparation (diluted if necessary) and sufficient 0.2 M phosphate buffer, pH 7.2, to make 1 ml. were pipetted into a 15 x 100 mm. tube. 100 mg. of collagen were added followed by 4 ml. of distilled water. The tube was shaken and then incubated at 37° C. for 3 hours. At the end of the incubation the contents of the tube were poured into a 15 ml. centrifuge tube and centrifuged at 3,000 r.p.m. for 15 minutes. A 1:10 dilution was made of the supernatant. Nitrogen was determined on 0.6 ml. of this dilution by the micro-Kjeldahl method. The milligrams of soluble nitrogen in the total reaction mixture (5 ml.) was calculated and multiplied

by the factor 5.85 to convert to milligrams of collagen solubilized. The activity was expressed as the milligrams of collagen solubilized per milligrams of enzyme preparation nitrogen in 3 hours at 37° C. from 100 mg. of collagen. This has been abbreviated in this paper as "CV," collagen value.

Determination of Amidase Activity. Amidase activity was determined by use of the micro-diffusion technique of Conway (8). A series of Conway vessels was prepared in the following manner: The outer rim of the vessel was greased with a thin film of stopcock grease. One ml. of 1 N HCl was pipetted into the center well of the vessel. Into the outer chamber were pipetted 1 ml. of saturated K_2CO_3 solution, a drop of phenolphthalein solution and enough water to make the final volume approximately 2 ml. after addition of the reaction mixture aliquot. At the desired time an aliquot of 0.25 ml. to 1 ml. of the reaction mixture was pipetted into the outer chamber and the glass cover was placed over the vessel. The contents of the outer chamber were mixed by gently rotating the vessel. Thorough mixing was indicated when the pink color of the phenolphthalein was evenly distributed in the outer chamber. After 100 minutes 0.6 ml. of the center well solution was withdrawn, nesslerized as previously described, and the nitrogen determined from a standard curve. Controls in the absence

of enzyme were run to determine any spontaneous hydrolysis of the amides.

The standard curve was prepared using standard solutions of ammonium sulfate which was previously dried at 110° C. for 2 hours. The addition of 0.2 ml. of digestion mixture, used in the determination of the nitrogen content of enzyme preparations, did not change the standard curve. A standard curve was made up each day that nitrogen determinations were run. The standard curve did not change from day to day. The data for the standard curve are given in Table I; the curve is plotted in Figure 1.

Table I

Nitrogen Standard Curve - Data

1 ml. standard $(NH_4)_2SO_4$ = 25 µg. nitrogen. 4 ml. Nessler's reagent used; 5 ml. of 2 N NaOH used. Total volume: 19.6 ml. Evelyn colorimeter Filter No. 515.

Standard $(NH_4)_2SO_4$ Solution	H_2O	N	%T	O.D.
ml.	ml.	µg.		
0 blank	10.6	0	set 100	0.000
0.5	10.1	12.5	86	0.066
1.0	9.6	25.0	75	0.125
1.5	9.1	37.5	65	0.187
2.0	8.6	50.0	56	0.252
2.5	8.1	62.5	49	0.310
3.0	7.6	75.0	43	0.367
3.5	7.1	87.5	37	0.432
4.0	6.6	100.0	31	0.509

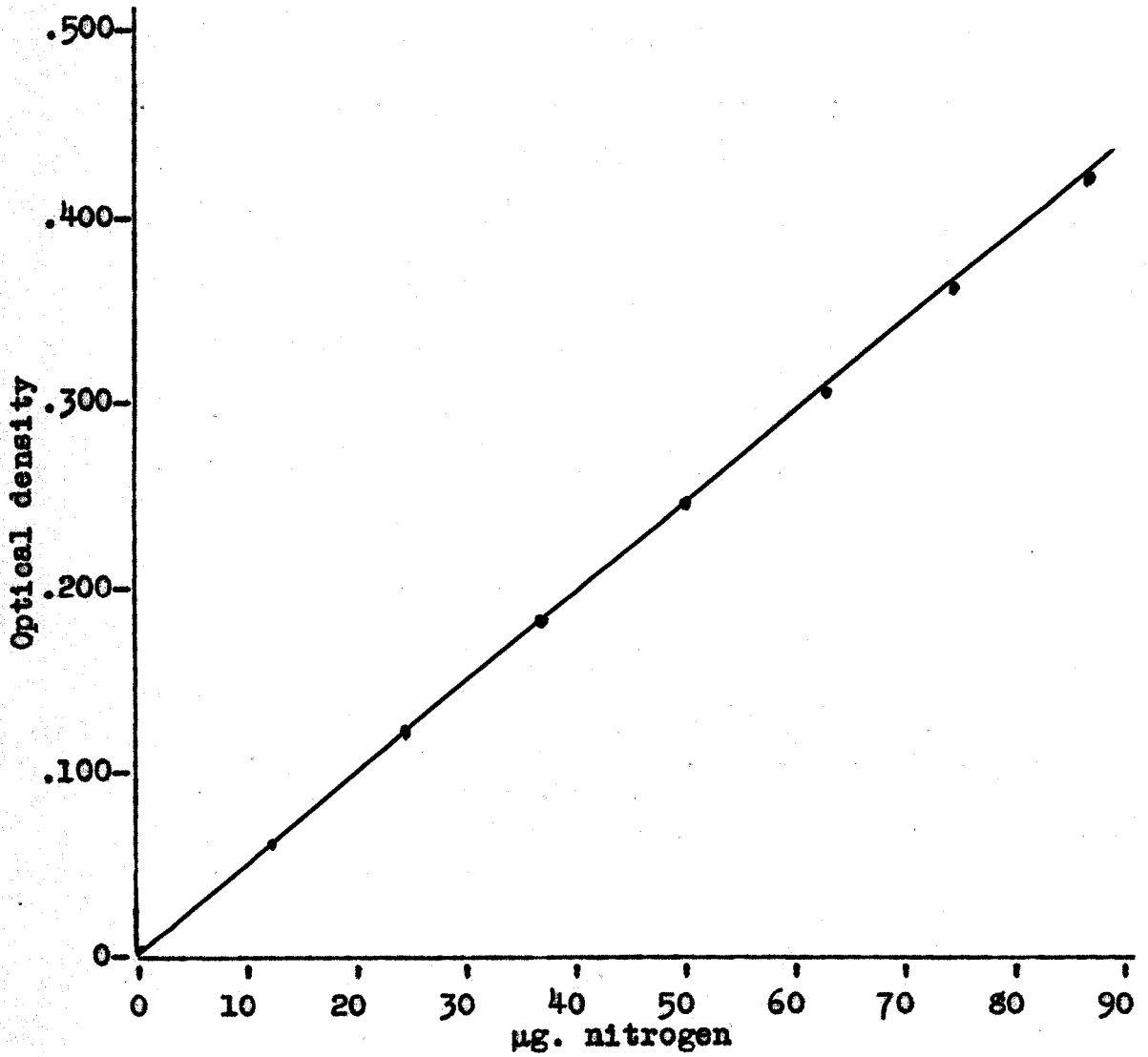

Figure 1. Nitrogen Standard Curve

Determination of Esterase Activity. Esterase activity was determined by applying the colorimetric hydroxamic acid method of Hestrin (9) to determine the residual ester in the reaction mixture. This method depends on the conversion of the unhydrolyzed ester into the corresponding hydroxamic acid by reaction with hydroxylamine in alkaline medium. A ferric-hydroxamide complex is then formed with ferric chloride in acid medium. This complex is deeply colored, ranging from dark brown to purple. Within limits of concentration the intensity of the color of the complex obeys the Beer-Lambert law. Other acid derivatives such as acyl phosphates, anhydrides, amides, and acyl halides give this reaction but under different conditions of pH. Fortunately the reaction is very rapid and occurs at room temperature. The colored complex is somewhat sensitive to pH changes. For this reason all reagents should be C. P. and all volumes should be measured accurately.

Reagents:-

2 M hydroxylamine hydrochloride solution

3.5 N sodium hydroxide solution

4 N hydrochloric acid solution

0.74 M ferric chloride in 0.1 N hydrochloric acid

The hydroxylamine hydrochloride solution is stable for several months if stored in the cold room. Equal volumes of the hydroxylamine hydrochloride solution and the 3.5 N NaOH solution are mixed just before use. This solution is the alkaline hydroxylamine solution; it is stable for about 3 hours at room temperature.

To determine esterase activity the following procedure was used. At zero time and at desired intervals during the enzyme reaction 0.5 ml., 1 ml., or 2 ml. aliquots of the reaction mixture were pipetted into 4 ml. of the alkaline hydroxylamine reagent in an Evelyn colorimeter tube, and enough buffer was added to make the total volume 6 ml. in each case. After 3 minutes, 2 ml. of 4 N HCl solution were added followed by 2 ml. of the 0.74 M ferric chloride solution. The color of the ferric-hydroxamide complex was read immediately against a reagent blank in an Evelyn colorimeter with filter No. 540 and at the 6 ml. level. The blank was made up in the same manner as the other solutions except that the order of addition of the NaOH solution and the HCl solution was reversed. No hydroxamic acid is formed from the esters in acid medium. Concentrations of residual ester in the reaction mixture were determined from a standard curve, described below. Controls were run in the absence of enzyme to determine any spontaneous hydrolysis of the ester. The choice of the size of the reaction mixture

aliquot taken was determined by the concentration of ester in the reaction mixture. Hestrin suggested that for concentrations of ester too high to be read in the colorimeter, the solution of the complex can be diluted with 0.074 M ferric chloride solution. Better results were obtained, however, by taking a 0.5 ml. or 1 ml. sample instead of a 2 ml. sample and making the solution up to volume with buffer.

Standard Curve:-

The standard curve for arginine methyl ester dihydrochloride is shown in Figure 2. The method for its construction applies to all of the esters used. An 0.025 M solution of arginine methyl ester dihydrochloride was prepared and dilutions of it were made to obtain solutions with concentrations ranging from 0.025 M to 0.00125 M. Two ml. aliquots of each solution were taken and the color developed as described above. The ferric chloride solution was added to only three solutions at a time and these were then read in the colorimeter before developing the color of the next three, and so on. The reason for this was that the color of the complex faded slightly upon standing. Figure 2 demonstrates thoroughly the linearity between concentration of ester and optical density. Once this linearity was established it was not necessary to construct a standard

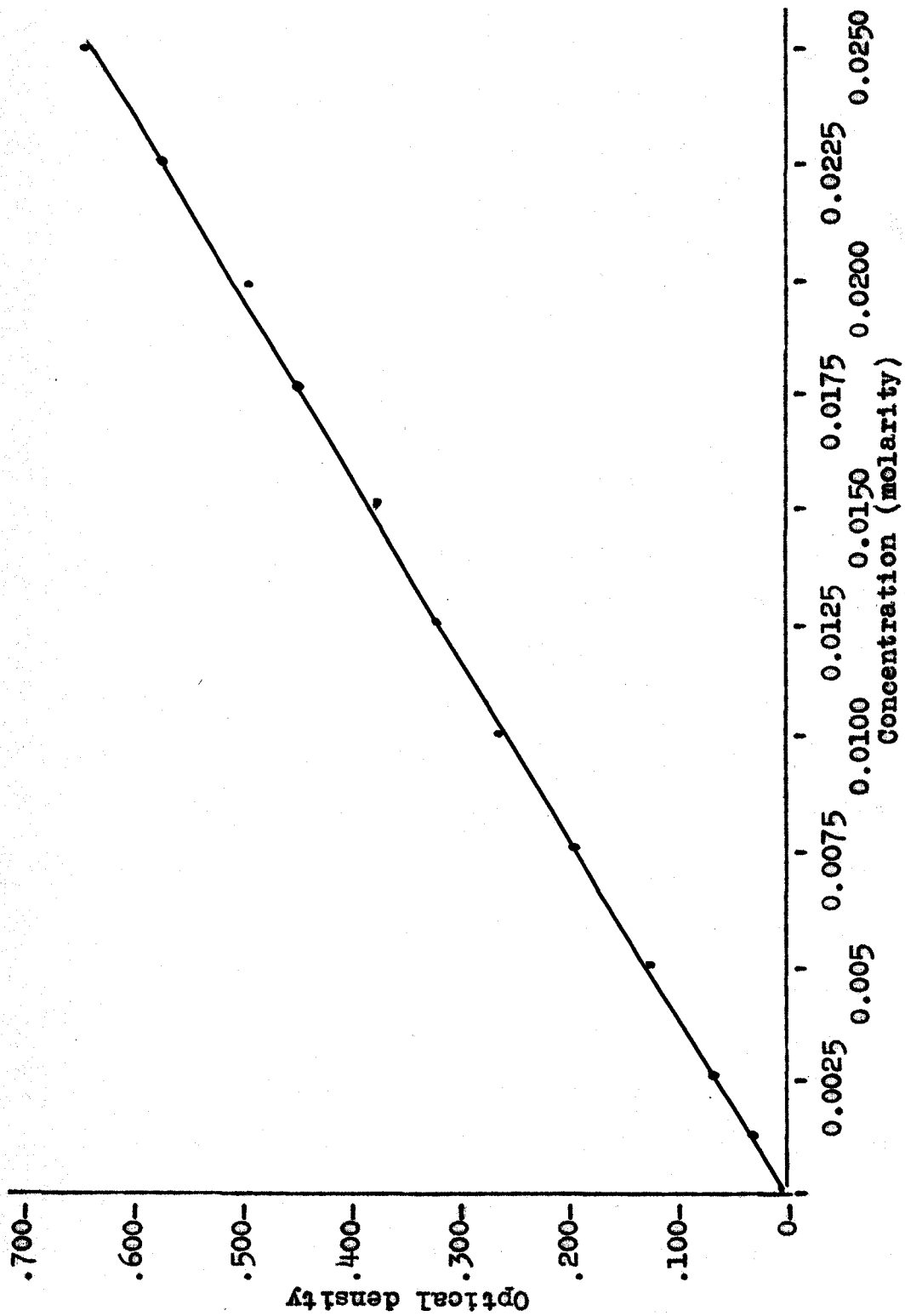

Figure 2. Arginine Methyl Ester Standard Curve

curve for each enzyme experiment. The concentration of residual ester could be calculated by proportionality, the optical density of the zero time sample representing the initial substrate concentration.

RESULTS

The Activity of Collagenase Preparations toward Synthetic Substrates

It was pointed out in the introduction that the nature in which collagenase attacks native collagen is not known. The reasons for this are: 1) the exact composition of the collagen molecule has not yet been determined; 2) collagenase has not yet been purified to the degree that it is known to be free from other proteolytic enzymes. Indeed, collagenase itself may not be a true proteinase. In all probability, the use of collagen as a substrate for crude collagenase preparations does measure collagenase activity, but it does not necessarily measure collagenase purity with respect to other proteolytic enzymes present in the enzyme preparation. This is true provided that at least one of the contaminating proteolytic enzymes remains in the enzyme preparation while other nitrogenous substances are eliminated. Thus as purification progresses, the ratio of collagenase to the contaminating enzyme will always be the same per unit of enzyme preparation, although both will be increasing in quantity.

It was observed that the collagenase preparations studied hydrolyzed gelatin as well as collagen. It was also

observed that although the activity toward collagen could be destroyed by heating the enzyme preparation at 50° C., there remained a residual activity toward gelatin. When cysteine was added to the original enzyme preparation the activity toward collagen was decreased and there were varying degrees of activity toward gelatin. These observations certainly indicated the presence of a contaminating proteinase in the collagenase preparation. It would be possible, by a complicated series of experiments, to determine whether the collagenase itself hydrolyzed gelatin, and thus devise a method for determining the purity of collagenase preparations with respect to other proteinases. However, methods of measuring enzyme activity based on protein substrates are not always reliable for reasons already discussed. The use of simple synthetic substrates offers rapid and more reliable methods for determining enzyme activity.

At the beginning of this investigation, very active collagenase preparations had been made which were thought to be free from contaminating proteinase as measured by the hydrolysis of gelatin.

It should be mentioned at the offset that no attempts were made to determine the "absolute" purity of the enzyme preparations by physical means. Therefore the use of the terms collagenase and proteinase throughout this paper

will be understood to mean collagenase preparations and proteinase preparations.

Synthetic Substrate Specificity of Collagenase. This series of experiments was done for the purpose of "screening" the various amino acid derivatives in order to determine the specificity, if any, of collagenase toward synthetic substrates. Reaction mixtures were made up as follows: An amount of stock enzyme preparation was diluted with buffer such that equal volumes of the diluted enzyme solution and substrate solution gave the desired concentration of enzyme. Naturally, substrate solutions were also made up in twice the desired final concentration. These solutions were incubated separately at the desired temperature for 5 minutes. Equal volumes of enzyme solution and substrate solution were then mixed and an interval timer started. Controls were prepared by mixing equal volumes of substrate solution and buffer. For the esters, a 2 ml. sample was taken at once and treated as described under Esterase Activity, page 18. This represented the zero time sample and the optical density, O. D., of its ferric-hydroxamide complex represented the initial substrate concentration. Two-ml. samples were then taken at desired intervals and treated in the same manner. Any appreciable decrease in O. D. with respect to the control indicated a

decrease in the residual ester in the reaction mixture and therefore indicated hydrolysis of the ester. For the amides, aliquots of the reaction mixture were taken at desired intervals and "Conwayed" as described on page 15. The detection of any appreciable amount of NH_3 over and above the control indicated hydrolysis of the amides. The conditions of the experiments and results are tabulated in Table II. The overall results are given in Table III. For the purpose of brevity, the substrates have been abbreviated. These abbreviations are given in Table III.

The Activity of Collagenase on BAEE. Having found several synthetic substrates toward which collagenase possessed activity, it was of interest to determine whether the enzymatic reactions adhered to some type of orderly kinetics. This property of the enzyme-substrate system would be a necessity for the development of a method of measuring enzyme purity and for the characterization of the system with respect to pH optimum, activation or inhibition behavior, and heat stability. As a rule, the reactions of enzymes and simple substrates follows simple zero order kinetics or apparent first order kinetics or a composite of both. Neurath and Schwert have given an excellent review (10) on enzyme reaction kinetics. BAEE was chosen as the substrate for this study because it could be prepared in very pure form.

Table II

Synthetic Substrate Specificity of Collagenase

All reactions were carried out in phosphate buffer.

Substrate	Concn. of Substrate M	Concn. of Enzyme*	Concn. of Buffer M	pH	Temp. °C.	Time min.	%T	O.D.
AME	0.0125	0.018	0.05	5.8	36	0	29	0.538
						60	30	0.523
Control		0				60	31	0.509
AME	0.0125	0.045	0.05	7.2	37	0	26	0.585
						30	28	0.552
						120	32	0.495
Control		0				30	27	0.569
						120	32	0.495
AME	0.005	0.045	0.05	7.2	37	0	58	0.237
						30	59	0.229
						120	63	0.201
Control		0				120	63	0.201
AME	0.005	0.022	0.1	7.2	37	0	62	0.208
						60	64	0.194
						120	67	0.174
Control		0				120	68	0.168
LyME	0.0125	0.018	0.05	5.8	36	0	32	0.495
						60	31	0.509
						120	31	0.509
Control		0				120	32	0.495
LyME	0.0125	0.045	0.05	7.2	37	0	28	0.552
						30	30	0.523
						120	34	0.469
Control		0				120	35	0.456
LyME	0.005	0.022	0.1	7.2	37	0	64	0.194
						120	68	0.168
Control		0				120	68	0.168
BABE	0.005	0.037	0.05	7.2	28	0	21	0.678
						60	34	0.469
						120	41	0.387
Control		0				60	22	0.658
						120	20	0.699

* mg. enzyme preparation nitrogen/ml. of test solution

Table II (Cont.)

Substrate	Concn. of Substrate M	Concn. of Enzyme*	Concn. of Buffer M	pH	Temp. °C.	Time min.	%T	O.D.
BABE	0.005	0.030	0.05	7.2	36	0	17	0.770
						15	40	0.398
						45	80	0.097
						60	90	0.046
Control		0				60	17	0.770
BABE	0.005	0.021	0.1	7.2	36	0	28	0.552
						20	36	0.444
						35	50	0.301
						50	70	0.155
						65	89	0.051
Control		0				65	27	0.569
BAEE	0.01	0.030	0.05	7.2	36	0	28	0.552
						15	37	0.432
						45	44	0.357
						75	52	0.284
Control		0				75	28	0.552
BAEE	0.003	0.020	0.1	7.2	36	0	55	0.260
						10	72	0.143
						15	80	0.097
						23	87	0.061
Control		0				23	56	0.252
BAIPE	0.003	0.020	0.1	7.2	36	0	63	0.201
						10	70	0.155
						15	72	0.143
						23	77	0.114
Control		0				23	62	0.208
BAA	0.0125	0.035	0.1	7.2	37	0	100	0.000
						10	82	0.086
						30	63	0.201
						60	56	0.252
Control		0				30	98	0.009
						60	96	0.018

* mg. enzyme preparation nitrogen/ml. of test solution

Table II (Cont.)

Substrate	Concn. of Substrate M	Concn. of Enzyme*	Concn. of Buffer M	pH	Temp. °C.	Time min.	%T	O.D.
ProBE	0.0125	0.037	0.05	5.8	36	0	40	0.398
						120	42	0.377
Control		0				120	41	0.387
ProBE	0.0125	0.037	0.05	7.2	36	0	42	0.377
						120	45	0.347
Control		0				120	44	0.357
GGEE	0.006	0.037	0.05	5.8	36	0	10	1.000
						120	10	1.000
Control		0				120	10	1.000
GGEE	0.006	0.037	0.05	7.2	36	0	10	1.000
						120	10	1.000
Control		0				120	10	1.000
LeuME	0.0125	0.037	0.05	7.2	36	0	27	0.569
						120	26	0.585
Control		0				120	26	0.585
PAEE	0.025	0.035	0.1	7.2	37	0	12	0.921
						60	13	0.886
Control		0				60	12	0.921

* mg. enzyme preparation nitrogen/ml. of test solution

Table III

Synthetic Substrate Specificity of Collagenase

Substrate	Abbreviation	Activity of Collagenase
L-Arginine methyl ester dihydrochloride	AME	-
L-Lysine methyl ester dihydrochloride	LyME	-
α-Benzoyl-L-arginine benzyl ester hydrochloride	BABE	+
α-Benzoyl-L-arginine ethyl ester hydrochloride	BAEE	+
α-Benzoyl-L-arginine isopropyl ester hydrochloride	BAIPE	+
α-Benzoyl-L-argininamide hydrochloride monohydrate	BAA	+
L-Proline benzyl ester hydrochloride	ProBE	-
Glycylglycine ethyl ester hydrochloride	GGEE	-
L-Leucine methyl ester hydrochloride	LeuME	-
DL-Phenylalanine ethyl ester hydrochloride	PAEE	-
L-Leucylglycylglycine	LeuGG	-*
Glycylglycine	GG	-*
Glycylglycylglycine	GGG	-*

* These substrates were used to determine any peptidase activity which might arise as a result of autolysis of the bacterial cells.

The data for the reaction of collagenase on BAEE are tabulated in Tables IV and V. The results are plotted in Figure 3. It can be seen that from 0.002 M to 0.01 M initial substrate concentration the reaction is zero order. The zero order reaction rate constant is independent of initial substrate concentration as it should be. The inset shows that enzyme activity is proportional to enzyme concentration.

The Activity of Collagenase on BAA. Several collagenase preparations were tested for their activity on BAA. The course of hydrolysis appeared to be first order but good agreement of first order constants could not be obtained. Table VI shows the hydrolysis of BAA by four different collagenase preparations. Different initial substrate concentrations were used in order to observe whether any order of kinetics would be indicated. With later preparations the results shown in Table VII were obtained. The course of reaction was at first zero order and then dropped off in the rate of hydrolysis to first order kinetics. This type of kinetics has been observed for many enzymes and has been interpreted according to the classical Michaelis-Menten enzyme reaction kinetics (11). The kinetics of the collagenase-BAA system were not studied further at this time because it was first desirable to determine whether the enzyme was stable at 37° C. over such long periods of reaction time.

Table IV

Effect of Initial Substrate Concentration on Collagenase-BAEE System

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Enzyme concentration was 0.016 mg. enzyme preparation nitrogen per ml. of test solution.

Initial Substrate Conen. (a)	Time	%T	O. D.	Moles per Liter Hydrolyzed	K x 10^4*
M	min.				
0.002	0	65	0.187	---	---
	2	68	0.168	0.0002	1.0
	4	71	0.149	0.0004	1.0
	6	74	0.131	0.0006	1.0
	8	77	0.114	0.0008	1.0
	10	81	0.092	0.0012	1.2
	14	84	0.076	0.0012	
0.005	0	38	0.420	---	---
	5	43	0.367	0.0006	1.20
	10	49	0.310	0.0012	1.20
	15	54	0.268	0.0018	1.20
	20	61	0.215	0.0024	1.20
	25	69	0.161	0.0032	
	30	74	0.131	0.0034	
	35	82	0.086	0.0040	
	40	86	0.066	0.0042	
	45	90	0.046	0.0045	
0.01	0	60	0.222	---	---
	15	66	0.181	0.0018	1.2
	22	69	0.161	0.0027	1.2
	30	72	0.143	0.0036	1.2
	38	75	0.125	0.0044	1.2
	46	79	0.102	0.0054	1.2
	56	83	0.081	0.0063	

* K = moles per liter hydrolyzed per minute.

Table V

Effect of Enzyme Concentration on Collagenase-BAEE System

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Initial substrate concentration was 0.005 M. Enzyme concentrations are in mg. enzyme preparation nitrogen per ml. of test solution.

Enzyme Conen. (e)	Time	%T	O. D.	Moles per Liter Hydrolyzed	K x 10^4*	K/e x 10^3
M	min.					
0.0244	0	38	0.420	---	---	---
	4	43	0.367	0.0006	(1.5)	---
	8	50	0.301	0.0014	1.7	
	12	59	0.229	0.0023	1.9	7.4
	16	68	0.168	0.0030	1.9	
	20	76	0.119	0.0036	1.8	
	24	82	0.086	0.0040	1.7	
0.016	0	38	0.420	---	---	
	5	43	0.367	0.0006	1.2	
	10	49	0.310	0.0012	1.2	
	15	54	0.268	0.0018	1.2	7.3
	20	61	0.215	0.0024	1.2	
	25	69	0.161	0.0032		
	30	74	0.131	0.0034		
	35	82	0.086	0.0040		
0.0325	0	38	0.420	---	---	
	5	48	0.319	0.0012	2.4	
	10	63	0.201	0.0026	2.6	7.6
	15	76	0.119	0.0036	2.4	
	20	89	0.051	0.0044		
	25	89	0.051			

* K = moles per liter hydrolyzed per minute.

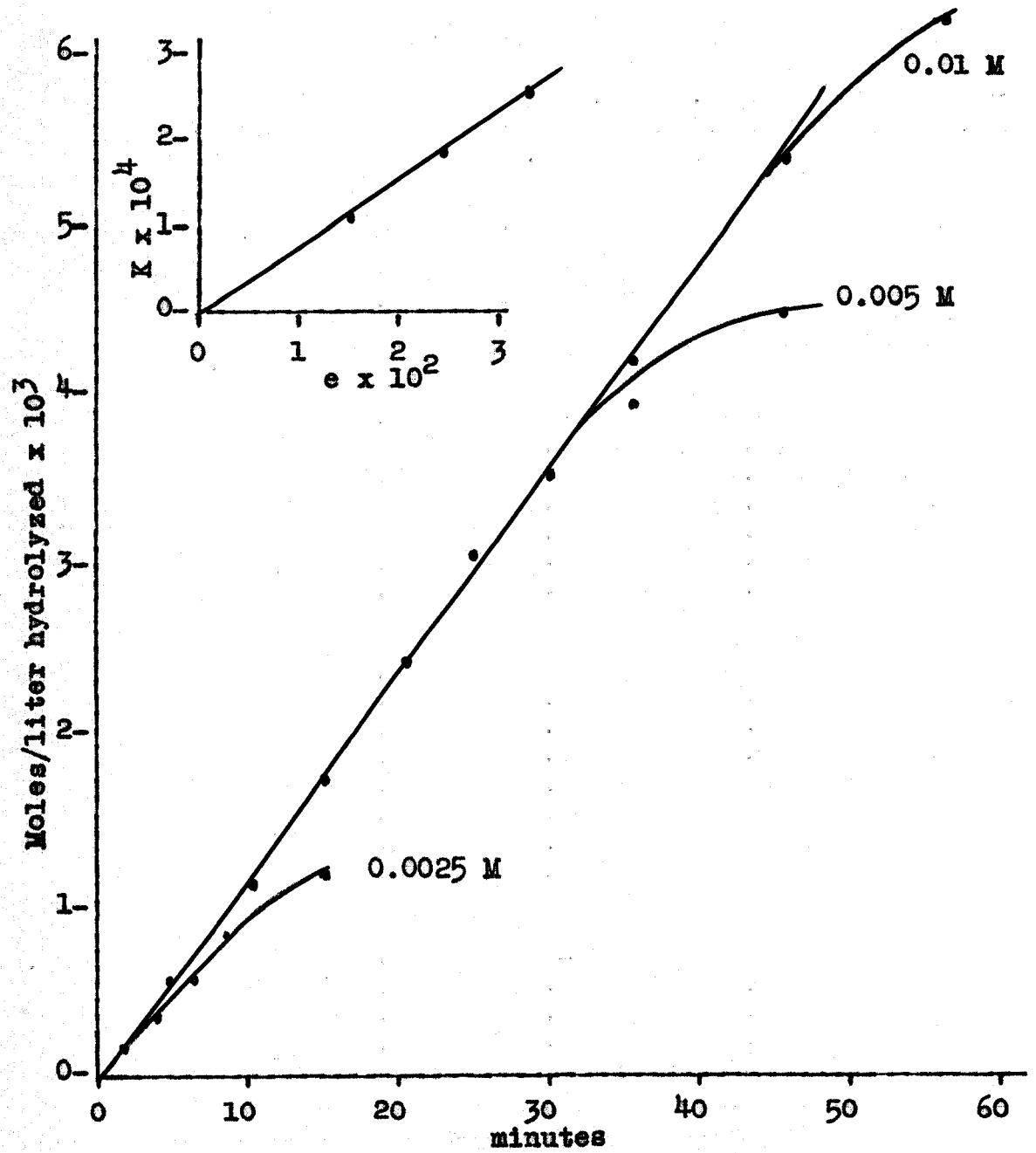

Figure 3. Hydrolysis of BAEE by Collagenase

Table VI

Hydrolysis of BAA by Various Collagenase Preparations

Reactions were carried out at 37° C. in 0.1 M phosphate buffer.

Enzyme Preparation	a	t	v	O. D.	N	x	K
I	0.05	15	0.3	0.051	10	0.0039	0.0027
		30	0.3	0.092	18	0.0071	0.0023
		45	0.15	0.066	13	0.0095	0.0020
		60	0.15	0.092	18	0.0143	0.0025
		120	0.15	0.108	22	0.0174	
II	0.0125	10	0.5	0.086	17	0.0041	0.017
		20	0.5	0.149	30	0.0071	0.018
		30	0.5	0.201	40	0.0095	0.020
		45	0.5	0.244	49	0.0115	0.024
		60	0.5	0.252	50	0.0119	0.021
		90	0.3	0.155	31	0.0123	
III	0.025	15				0.0048	0.006
		30				0.0078	0.0052
		45				0.0106	0.0052
		60				0.0139	0.0057
		120				0.0181	
IV	0.005	3	1.0	0.061	12	0.0014	0.050
		6	1.0	0.097	19	0.0023	0.045
		9	1.0	0.149	30	0.0036	0.060
		12	1.0	0.181	36	0.0043	0.070
		15	1.0	0.194	38	0.0046	0.073
		20	1.0	0.208	42	0.005	

a = initial substrate concentration in molarity
t = time in minutes
v = volume of reaction mixture "Conwayed" in ml.
N = micrograms of nitrogen obtained from standard curve, page 17.
x = moles per liter hydrolyzed

$$K = 1/t \log \frac{a}{a - x}$$

Table VII

Effect of Enzyme Concentration on Collagenase-BAA System

Reactions were carried out at 37° C. in phosphate buffer, pH 7.2. Initial substrate concentration was 0.0125 M. (Enzyme preparation different from those in Table VI.)

e	t	v	%T	O. D.	N	x	K x 10^4	K/e
0.050	5	0.5	90	0.046	9	0.002	4.0	
	10	0.5	81	0.092	18	0.004	4.0	0.0080
	15	0.5	73	0.137	27.5	0.0065	4.3	
	20	0.5	67	0.174	35	0.008	4.0	
	25	0.5	63	0.201	40	0.0095		
	45	0.3	71	0.149	30	0.012		
0.0175	5	1.0	93	0.032	6	0.0007	1.4	
	10	1.0	87	0.061	12	0.0014	1.4	0.0080
	20	1.0	78	0.108	22	0.0026	1.3	
	30	1.0	73	0.137	28	0.0033		
	45	0.5	79	0.102	20	0.0048		
	60	0.5	76	0.119	24.5	0.0058		
	90	0.5	71	0.149	30	0.0071		
	120	0.5	70	0.155	31	0.0074		
	150	0.5	66	0.181	36	0.0085		
	180	0.5	65	0.187	38	0.009		

e = concentration of enzyme in mg. nitrogen per ml. test solution
t = time in minutes
v = volume of reaction mixture taken, in ml.
N = micrograms of nitrogen obtained from standard curve, page 17.
x = moles per liter hydrolyzed
K = moles per liter hydrolyzed per minute

The Effect of Preliminary Heating of Collagenase on the Collagenase-BAA System. It has been reported (6) that heating collagenase preparations at 50° C. destroyed 98% of the activity toward collagen but left some residual activity toward gelatin. It was of interest to determine if the activity of collagenase toward BAA was also destroyed by heat. Enzyme and buffer were incubated at 50° C. or at 45° C. for different periods of time. After the desired time, the enzyme tubes were plunged into an ice-water bath to bring the temperature down rapidly to 37° C. Then equal volumes of BAA solution were added and the tubes were incubated at 37° C. Aliquots were withdrawn at intervals and the NH_3 determined. Controls were run in which enzyme and buffer were incubated at 37° C. for time intervals corresponding to the incubation times at 50° C. The results are tabulated in Tables VIII and IX.

It can be seen from Table VIII that heating the enzyme preparation at 50° C. for 5 to 30 minutes resulted in about 30% inactivation. The residual activity toward BAA was nearly constant. The residual activity decreased upon heating the enzyme at 50° C. for 45 minutes. The table also shows that there is a slight decrease in activity upon increasing time of incubation at 37° C. This is shown by the controls. Table IX summarizes the results of a similar experiment in which the heat inactivation was carried out

Table VIII

Effect of Preliminary Heating of Collagenase at 50° C. on the Collagenase-BAA System

After heating at 50° C. for periods indicated, reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Enzyme concentration was 0.035 mg. N/ml. test solution. Initial substrate concentration was 0.0125 M.

Time of Incubation of Enzyme at 50° C. or Control at 37° C.	Time of Incubation of Reaction Mixture	v	%T	O. D.	N	x
min.	min.					
5	60	0.5	86	0.066	13	0.0031
5 (control)	60	0.5	61	0.215	43	0.010
10	60	0.5	87	0.061	12	0.0029
15	60	0.5	87	0.061	12	0.0029
20	60	1.0	75	0.125	25	0.003
20 (control)	60	0.5	63	0.201	40	0.0095
30	60	1.0	77	0.114	23	0.0027
45	60	1.0	84	0.076	16	0.0019
30	10	0.4	91*	0.041	4	0.0012
30 (control)	10	0.4	88*	0.056	5.5	0.0013
30	30	0.4	88*	0.056	5.5	0.0013
30 (control)	30	0.4	71*	0.149	15	0.0044
30	60	0.4	84*	0.076	7.5	0.0022
30 (control)	60	0.4	52*	0.284	28.7	0.0084
30	95	0.4	92	0.036	7	0.0021
30 (control)	95	0.4	70	0.155	31	0.0091

v = volume of reaction mixture taken, in ml.
N = micrograms nitrogen obtained from standard curve, page 17.
x = moles per liter hydrolyzed

* Samples nesslerized at half volume

Table IX

Effect of Preliminary Heating of Collagenase at 45° C. on the Collagenase-BAA System

After heating at 45° C. for periods indicated, reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Enzyme concentration was 0.035 mg.N/ml. test solution. Initial substrate concentration was 0.0125 M.

Time of Incubation of Enzyme at 45° C. or Control at 37° C.	Time of Incubation of Reaction Mixture	v	%T	O. D.	N	x
min.	min.					
5	60	0.5	69	0.161	32	0.0076
5 (control)	60	0.5	58	0.237	48.5	0.0115
10	60	0.5	77	0.114	23	0.0055
15	60	0.5	80	0.097	20	0.0048
20	60	1.0	65	0.187	38.5	0.0046
30	60	1.0	70	0.155	32	0.0038
45	60	1.0	75	0.125	25	0.003
30	60	1.0	67	0.174	35	0.0042
30 (control)	60	0.5	65	0.187	38.5	0.0092
30	90	1.0	64	0.194	39.5	0.0047
45	60	1.0	75	0.125	25	0.003
45 (control)	60	0.5	70	0.155	31	0.0074
60	60	1.0	78	0.108	22	0.0026
60 (control)	60	0.5	75	0.125	25	0.006
90	60	1.0	84	0.076	15	0.0018
90 (control)	60	1.0	60	0.222	44	0.0052

v = volume of test solution taken, in ml.
N = micrograms of nitrogen obtained from standard curve, page 17.
x = moles per liter hydrolyzed.

at 45° C. instead of at 50° C. After 45 minutes incubation at 45° C., the same residual activity was obtained as that observed after 20 minutes incubation at 50° C. The table again shows the decrease in activity upon increasing time of incubation at 37° C. (controls). Two conclusions can be drawn from the results of these experiments. First, the collagenase preparation was apparently contaminated with a "relatively" stable proteinase which hydrolyzes BAA. Second, the collagenase preparation was slowly inactivated at 37° C.

The Effect of $FeSO_4$ Plus Cysteine on the Heat Stable Component of the Collagenase Preparation. It has been reported (2) that Cl. histolyticum elaborates an extracellular proteinase which hydrolyzes gelatin and is maximally activated by Fe^{++} plus cysteine. Although earlier studies of the activity of crude collagenase preparations toward collagen and gelatin had indicated the presence of a heat stable proteinase, no attempt was made to identify this proteinase with the Fe^{++}-cysteine activatable proteinase reported. Since the results of heat inactivation on the collagenase-BAA system indicated the presence of a contaminating proteinase, it was of interest to determine whether the activity of the heat treated preparation was increased by Fe^{++} plus cysteine.

The results are shown in Table X for both BAA and BAEE. For this experiment, the collagenase solutions were heated for 10 minutes at 50° C. to destroy "collagenase" activity. Then the activator ($FeSO_4$ plus cysteine) was added to two of the enzyme solutions and buffer to the other two. The tubes were incubated at 37° C. to insure ample activation time. Next, the substrate, BAA or BAEE, was added and samples taken in 15 minutes. Controls, in the absence of enzyme but with activator, were run.

It can be seen from Table X that the heat treated collagenase preparation was highly activated by $FeSO_4$ plus cysteine.

The Effect of $FeSO_4$ Plus Cysteine on the Heat Inactivated Collagenase Preparation with Respect to Arginine Methyl Ester (AME). Since the heat treated collagenase preparation gave a residual activity toward BAA and BAEE, it was of interest to determine whether the heat treated enzyme would now be active toward AME. Also, since the residual activity toward BAA and BAEE was increased by the addition of $FeSO_4$ plus cysteine, it was desirable to determine whether the addition of this activator would now effect an activation toward AME.

Reaction mixtures and controls were prepared as follows: Enzyme and buffer were incubated at 50° C. for 10 minutes to inactivate "collagenase." The tubes were then

Table X

Effect of $FeSO_4$ Plus Cysteine on the Heat Stable Component of the Collagenase Preparation

After heating enzyme at 50° C. for 10 minutes, reactions were carried out at 37° C. in phosphate-citrate buffer (McIlvaine), pH 7.2. Enzyme concentration during heat inactivation was 0.070 mg. N/ml. solution. Enzyme concentration during reaction with substrate was 0.035 mg. N/ml. test solution. Initial substrate concentration was 0.0125 M for BAA and 0.005 M for BAEE. Activator concentration was 0.008 M in cysteine and 0.002 M in $FeSO_4$.

	v	%T	O. D.	N	x
			BAA		
No activator added	1.0	81	0.092	18	0.002
Activator added	1.0	32	0.495	100	0.012
Control: activator but no enzyme	1.0	98	---	--	---
			BAEE		
Initial substrate concentration		55	0.260		---
No activator added		68	0.168		0.002
Activator added		100	0.000		0.005
Control: activator but no enzyme		56	---		---

v = volume of test solution taken, in ml.
N = micrograms of nitrogen
x = moles per liter hydrolyzed

plunged into an ice-water bath to bring the temperature down to 37° C. Activator was added and the tubes incubated at 37° C. for 30 minutes to insure sufficient activation time. Then AME solution was added and the tubes incubated at 37° C. for 15 minutes and residual ester determined. Three sets of controls were prepared for the experiment. In one control, enzyme and buffer were incubated at 50° C. for 10 minutes and treated in the same manner as the reaction mixture tubes above except that buffer was added in place of activator. For the second control, enzyme and buffer were incubated at room temperature so as not to inactivate the collagenase. Activator was added to this control. For the third control, enzyme and buffer were incubated at room temperature and buffer was added in place of activator. Appropriate blanks were prepared for comparing the reaction and control tubes. The results are shown in Table XI. Only qualitative results are given. Any decrease in O. D. with respect to the control indicates hydrolysis of AME.

It can be seen from the table that AME was definitely hydrolyzed (nearly 100%) when activator was added, whether the enzyme was previously heated or not. There was no activity toward AME in the absence of activator. This is still evidence that there is an activatable proteinase in the collagenase preparation. The fact that there is no residual activity toward AME without activator can possibly

Table XI

Effect of $FeSO_4$ Plus Cysteine on Heat Inactivated Collagenase Preparation with Respect to AME

After addition of substrate (AME), reactions were carried out at 37° C. in phosphate-citrate buffer (McIlvaine), pH 7.2. Enzyme concentration in final reaction mixtures was 0.035 mg. N/ml. test solution. Initial substrate concentration was 0.005 M. Activator concentration in reaction mixtures was 0.008 M in cysteine and 0.002 M in $FeSO_4$.

Tube*	Heat Inactivation Time min.	Activator	%T	O. D.
Initial Substrate Concentration	--	added	40	0.398
Test solution	10	added	95	0.022
Control 1	10	not added	39	0.409
Initial Substrate	--	not added	39	0.409
Control 2	0	added	93	0.032
Control 3	0	not added	39	0.409

* See text for explanation.

be explained by a much less affinity of the proteinase for AME as compared to its affinity for BAA or BAEE. This was later shown to be the case in studies of the proteinase. Furthermore, the finding of a synthetic substrate which is hydrolyzed by the proteinase but not by collagenase affords a very simple method for the detection of proteinase in collagenase preparations.

The Activity of Proteinase Preparations Toward Synthetic Substrates

Since the studies on the activity of collagenase preparations with respect to synthetic substrates indicated the presence of a contaminating proteinase, it was desirable to study the properties of this proteinase. A Cl. histolyticum proteinase has been reported in the literature (2, 3,4,5,6) and has been obtained (6) from the strain of Cl. histolyticum employed in this laboratory. Heretofore the proteinase has been studied with respect to protein substrates only.

This study deals with the use of synthetic substrates for the characterization of Cl. histolyticum proteinase.

The Activity of Proteinase Preparations toward Collagen. In order to determine whether the proteinase preparations contained any "collagenase" as indicated by the hydrolysis of collagen, collagenase activity was run on all preparations according to the method described on page 14. The results of a typical experiment are shown in Table XII. In order to determine whether collagen adsorbed the proteinase, activity toward AME was also run on the supernatants after removal of the collagen.

Table XII

The Activity of Proteinase Toward Collagen

Enzyme concentration was 0.2 mg. N in total reaction mixture (5 ml.). Activator was cysteine, 0.003 M. Temperature of incubation with collagen was 37° C.

Tube	Heated at 50° C.	Activator (cysteine)	%T	O. D.	CV*
	min.				
1	0	not added	97	0.013	< 3
2	10	not added	97	0.013	< 3
3	0	added	95	0.022	< 3
4	10	added	95	0.022	< 3

AME Activity of Supernatants

Supernatant	Activator (Cysteine)	%T	O. D.
Initial Substrate Concentration	not added	22	0.658
Initial Substrate Concentration	added	22	0.658
1	not added	22	0.658
1	added	55	0.260
2	not added	22	0.658
2	added	49	0.310
3	not added	21	0.678
3	added	52	0.284
4	not added	21	0.678
4	added	47	0.328

* The 3-hour CV of a moderately potent collagenase preparation is about 5,000.

It can be seen from the table that the proteinase preparation contained no appreciable collagenase activity. The proteinase was not adsorbed on the collagen as evidenced by the AME activity of the supernatants after activation. The results also indicate that there is a slight decrease in activity toward AME after incubation of the enzyme at 50° C. for 10 minutes.

None of the proteinase preparations employed in this study contained any appreciable collagenase activity.

Effect of Fe^{++} ($FeSO_4$ or $Fe(NH_4)_2(SO_4)_2$) alone or of Cysteine alone on Proteinase-AME Activity. Kocholaty (2) reported that proteinase is activated by cysteine and further activated by $FeSO_4$ plus cysteine. He observed in some instances activation by $FeSO_4$ alone and in other instances inhibition by this compound. Table XIII shows that in no case was proteinase activated toward AME by Fe^{++} alone. The table also shows that proteinase was activated by cysteine alone.

Table XIII

The Effect of Fe^{++} Alone or of Cysteine Alone on Proteinase-AME Activity

Reactions were carried out at 37° C. in phosphate-citrate buffer (McIlvaine), pH 7.2. Activator concentration was 0.002 M in ferrous salt and 0.008 M in cysteine.

	Activator	%T	O. D.
Initial Substrate Concentration	$Fe(NH_4)_2(SO_4)_2$	54	0.268
Test Solution	$Fe(NH_4)_2(SO_4)_2$	52	0.284
Initial Substrate Concentration	$FeSO_4$	51	0.292
Test Solution	$FeSO_4$	51	0.292
Initial Substrate Concentration	Cysteine	54	0.268
Test Solution	Cysteine	97	0.013

The Effect of Time of Incubation with Cysteine on Proteinase-AME Activity. Enzyme was incubated at room temperature with cysteine for varying periods of time. Then AME was added and the reaction mixtures incubated at 37° C. for 5 minutes and assayed for hydrolysis of the ester. The results are shown in Table XIV. It can be seen that the activity of the proteinase toward AME is independent of the time of activation.

Table XIV

Effect of Time of Incubation with Cysteine on Proteinase-AME Activity

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Enzyme concentration was 0.0125 mg. N/ml. test solution. Initial substrate concentration was 0.005 M. Cysteine, 1 mg./ml. test solution.

Time of Activation	%T	O. D.
min.		
0	83	0.081
1	84	0.076
5	84	0.076
10	82	0.086
15	84	0.076
30	83	0.081
Initial Substrate Concentration	54	0.268
Control: enzyme but no cysteine	54	0.268

The Effect of Cysteine Concentration on Proteinase-AME Activity. Table XV summarizes the results of the effect of cysteine concentration on proteinase-AME activity. It can be seen that from 1 mg. to 3 mg. of cysteine per ml. of test solution there was no change in activity. From 0.5 mg. to 0.1 mg. of cysteine per ml. of test solution there was a slight decrease in activity.

Table XV

Effect of Cysteine Concentration on Proteinase-AME Activity

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Enzyme concentration was 0.0125 mg.N/ml. test solution. Initial substrate concentration was 0.005 M.

Concn. of Cysteine	%T	O. D.
mg./ml. of test soln.		
0.1	76	0.119
0.25	80	0.097
0.5	84	0.076
1.0	85	0.071
2.0	85	0.071
3.0	85	0.071
Initial Substrate Concentration	54	0.268
Control: enzyme but no cysteine	53	0.276

The results of the previous experiments can be summarized as follows: The proteinase preparation contained no activity toward collagen. The proteinase was not activated by $FeSO_4$ or $Fe(NH_4)_2(SO_4)_2$ alone but was activated by cysteine alone. Maximum activation was obtained by cysteine concentrations from 1 mg. to 3 mg. per ml. of test solution. Activity was independent of time of activation.

Synthetic Substrate Specificity of Proteinase.

The results of the studies of collagenase preparations indicated that the proteinase hydrolyzed the esters and amide of α-benzoylarginine and arginine methyl ester (AME). It was noted that the proteinase in these preparations hydrolyzed AME while the collagenase did not. It was of interest to determine whether the proteinase displayed activity toward any other synthetic substrates.

The test solutions were prepared in the manner described on page 25 except that two reaction mixtures for each substrate were prepared, one with activator (cysteine) and the other without activator. Controls were run in the absence of enzyme to determine any spontaneous hydrolysis of the substrates. In no case was there observed any appreciable spontaneous hydrolysis of the substrates.

The results are summarized in Table XVI. It can be seen that the proteinase hydrolyzed only the esters and amide of α-benzoylarginine, arginine methyl ester, and, very slowly, lysine methyl ester. No hydrolysis of any of the substrates was observed unless the proteinase was first activated with cysteine. The fact that apparent proteinase activity toward BAA and BAEE was observed in the heat treated collagenase preparations without the addition of cysteine can possibly be explained by a partial activation of the proteinase by the collagenase present. Or, the proteinase may have

Table XVI

Synthetic Substrate Specificity of Cl. histolyticum Proteinase

Reactions carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2.

Substrate	Abbreviation	Activity of Proteinase*
L-Arginine methyl ester dihydrochloride	AME	+
α-Benzoyl-L-arginine benzyl ester hydrochloride	BABE	+
α-Benzoyl-L-arginine ethyl ester hydrochloride	BAEE	+
α-Benzoyl-L-arginine isopropyl ester hydrochloride	BAIPE	+
α-Benzoyl-L-argininamide hydrochloride monohydrate	BAA	+
L-Lysine methyl ester dihydrochloride	LyME	±**
DL-Phenylalanine ethyl ester hydrochloride	PAEE	-
N-Benzoyl-DL-Phenylalanine ethyl ester	BPAEE	-
N-Benzoyl-DL-phenylalaninamide	BPAA	-
L-Leucine methyl ester hydrochloride	LeuME	-

* Activity was determined for the activated and non-activated proteinase. For activation, cysteine was used in a concentration of 1 mg./ml. of test solution.
** Hydrolyzed very slowly.

Table XVI (contd.)

Substrate	Abbreviation	Activity of Proteinase*
L-Leucinamide hydrochloride	LeuA	-
L-Proline benzyl ester hydrochloride	ProBE	-
L-Prolinamide hydrochloride	ProA	-
Benzoylglycinamide	BGA	-
Glycylglycine ethyl ester hydrochloride	GGEE	-
L-Leucylglycylglycine***	LeuGG	-
Glycylglycine***	GG	-
Glycylglycylglycine***	GGG	-

* Activity was determined for the activated and non-activated proteinase. For activation, cysteine was used in a concentration of 1 mg./ml. of test solution.

*** These substrates were used to determine any peptidase activity which might arise as a result of autolysis of the bacterial cells.

been partially activated during the production of the collagenase preparations.

The Activity of Cysteine Activated Proteinase on AME. The reaction mixture, control for spontaneous hydrolysis of the ester, and blank were prepared as follows:

Test Solution:-

Twenty mg. of cysteine were placed in the reaction tube (25 x 200 mm.) and dissolved in 9.75 ml. of 0.1 M phosphate buffer, pH 7.2. 0.25 ml. of the proteinase preparation (1 ml. = 2 mg. nitrogen) were added and the tube was incubated at 37° C. for 5 minutes. This period of incubation brought the contents of the tube to 37° C. and also insured ample time for activation of the enzyme, although it was found that activity was independent of activation time. Then 10 ml. of 0.01 M AME (previously brought to 37° C.) were rapidly pipetted into the tube and time started. The tube was quickly shaken to mix the contents and a 2 ml. sample was withdrawn at once, pipetted into an Evelyn colorimeter tube containing 4 ml. of alkaline hydroxylamine solution, and treated as described on page 18. This was the zero time sample. The O. D. of the ferric-hydroxamide complex from this sample represented the initial substrate concentration. At desired time intervals, 2 ml. samples of the test solution were withdrawn and treated similarly.

Control:-

Five ml. of buffer at 37° C. containing 10 mg. of cysteine, and 5 ml. of 0.01 M AME at 37° C. were mixed and a 2 ml. sample withdrawn at once and treated as described above. This was the zero time control. Two ml. samples were withdrawn at desired intervals and treated similarly.

Blank:-

Ten mg. of cysteine were dissolved in 5 ml. of buffer. One ml. of this solution was pipetted into an Evelyn tube containing 4 ml. of alkaline hydroxylamine solution. After 3 minutes, 2 ml. of 4 N HCl were added then 1 ml. of 0.01 M AME and finally 2 ml. of ferric chloride solution. In this manner the blank corrects for the slight cysteine-hydroxylamine reaction which occurs when the test solution sample is pipetted into the hydroxylamine solution.

A standard curve was not constructed. The 0.01 M stock solution of AME was diluted to two or three different concentrations and 2 ml. aliquots of these were developed in the usual manner. The colors of these tubes were read and the linearity between optical density and concentration of AME verified. A standard curve over a large range of AME concentrations is given on page 21. It established the adherence to the Beer-Lambert law.

The test solutions, controls, and blanks for the other initial substrate concentrations of AME were prepared in the same manner as above except that the proper concentration of AME stock solution was used. The stock solution of substrate was naturally made up in double the final concentration desired in the test solution.

The data for the hydrolysis of AME by cysteine activated proteinase are shown in Table XVII. The data are plotted in Figure 4. It can be seen from the table that for any given initial substrate concentration, the course of hydrolysis was first order. However, the first order reaction rate constant decreased with increasing initial substrate concentration. For any given initial substrate concentration, the first order reaction rate constant divided by the enzyme concentration has been defined as the "proteolytic coefficient" at that initial substrate concentration and under the particular conditions of the reaction (12). It is known that in general enzymatic activity is proportional to enzyme concentration. Thus as enzyme concentration is increased, the first order constant is increased proportionately and the proteolytic coefficient, K/e, remains the same. This relationship holds provided that the order of the enzymatic reaction is not changed as enzyme concentration is varied. It should be emphasized that proteolytic coefficients must be compared at the same

initial substrate concentrations because the proteolytic coefficient increases as initial substrate concentration decreases.

In Table XVIII are summarized the data for the hydrolysis of AME at an enzyme concentration one half that used in the experiment tabulated in Table XVII. It can be seen by comparing the proteolytic coefficients in the two tables that the proteolytic coefficients for corresponding initial substrate concentrations are nearly the same. It will be noted that at the lower enzyme concentration (Table XVIII) the initial 15 minutes of hydrolysis was zero order for 0.005 M and 0.01 M initial substrate concentration. This zero order portion of the hydrolysis curve will give rise to low first order constants and these constants should not be considered in the average first order constant. The zero order hydrolysis of AME over so large an initial portion of the reaction was seldom observed in subsequent experiments. It is obvious that a large error would be obtained by disregarding this zero order portion of the curve and determining initial reaction velocity by extrapolation of the first order portion of the curve to zero time.

Table XVII

The Hydrolysis of AME by Cysteine Activated Proteinase

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Enzyme concentration, e, was 0.025 mg. enzyme preparation nitrogen/ml. of test solution. Concentration of cysteine was 1 mg./ml. test solution (0.008 M).

a	Time	%T	O. D.	x	K	K/e
	min.					
0.0025	0	54	0.268	---	---	---
	2	60	0.222	0.0004		
	5	72	0.143	0.0012	0.057	
	7	79	0.102	0.0015	0.057	
	10	86	0.066	0.0019	0.062	2.4
	12	88	0.056	0.002	0.058	
	15	92	0.036	0.0022	0.061	
0.005	0	26	0.585	---	---	---
	2	33	0.482	0.0009	(0.043)	
	5	42	0.377	0.0018	0.039	
	7	49	0.310	0.0023	0.041	
	10	59	0.229	0.003	0.040	1.6
	12	63	0.201	0.0033	0.040	
	15	69	0.161	0.0036	0.037	
	20	80	0.097	0.0042	0.040	
0.01	0	7	1.155	---	---	---
	2	9	1.046	0.0009	0.020	
	5	13	0.886	0.0023	0.023	
	7	15	0.824	0.0028	0.020	
	10	22	0.654	0.0043	0.024	
	12	25	0.602	0.0048	0.024	0.92
	15	30	0.523	0.0055	0.023	
	17	37	0.432	0.0063	0.025	
	20	40	0.398	0.0066	0.023	
	25	49	0.310	0.0073	0.022	

a = initial substrate concentration in molarity.
x = moles per liter hydrolyzed.

$$K = 1/t \log \frac{a}{a - x}$$

Table XVIII

The Hydrolysis of AME by Cysteine Activated Proteinase

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Enzyme concentration, e, was 0.0125 mg. nitrogen per ml. of test solution. Concentration of cysteine was 1 mg. per ml. of test solution

a	Time	%T	O. D.	x	K	K/e
	min.					
0.0025	0	56	0.252	---	---	---
	5	66	0.181	0.0007	0.028	
	10	74	0.131	0.0012	0.028	
	15	81	0.092	0.0016	0.029	2.24
	20	85	0.071	0.0018	0.028	
	25	90	0.046	0.002	0.028	
0.005	0	27	0.569	---	---	---
	5	33	0.482	0.0008	(0.015)	
	10	42	0.377	0.0017	(0.018)	
	15	52	0.284	0.0025	(0.020)	
	20	60	0.222	0.0030	0.020	
	25	67	0.174	0.0035	0.021	1.7
	30	74	0.131	0.0038	0.021	
	35	79	0.102	0.0041	0.021	
	40	82	0.086	0.0042	0.020	
	45	85	0.071	0.0044	0.020	
0.01	0	8	1.097	---	---	---
	5	10	1.000	0.001	(0.009)	
	10	13	0.886	0.0019	(0.009)	
	15	17	0.770	0.003	(0.010)	
	20	21	0.678	0.0038	0.010	
	25	26	0.585	0.0047	0.011	
	30	31	0.509	0.0054	0.011	
	35	40	0.398	0.0064	0.013	0.88
	40	43	0.367	0.0067	0.012	
	50	49	0.310	0.0072	0.011	

a = initial substrate concentration in molarity.
x = moles per liter hydrolyzed.

$$K = 1/t \log \frac{a}{a - x}$$

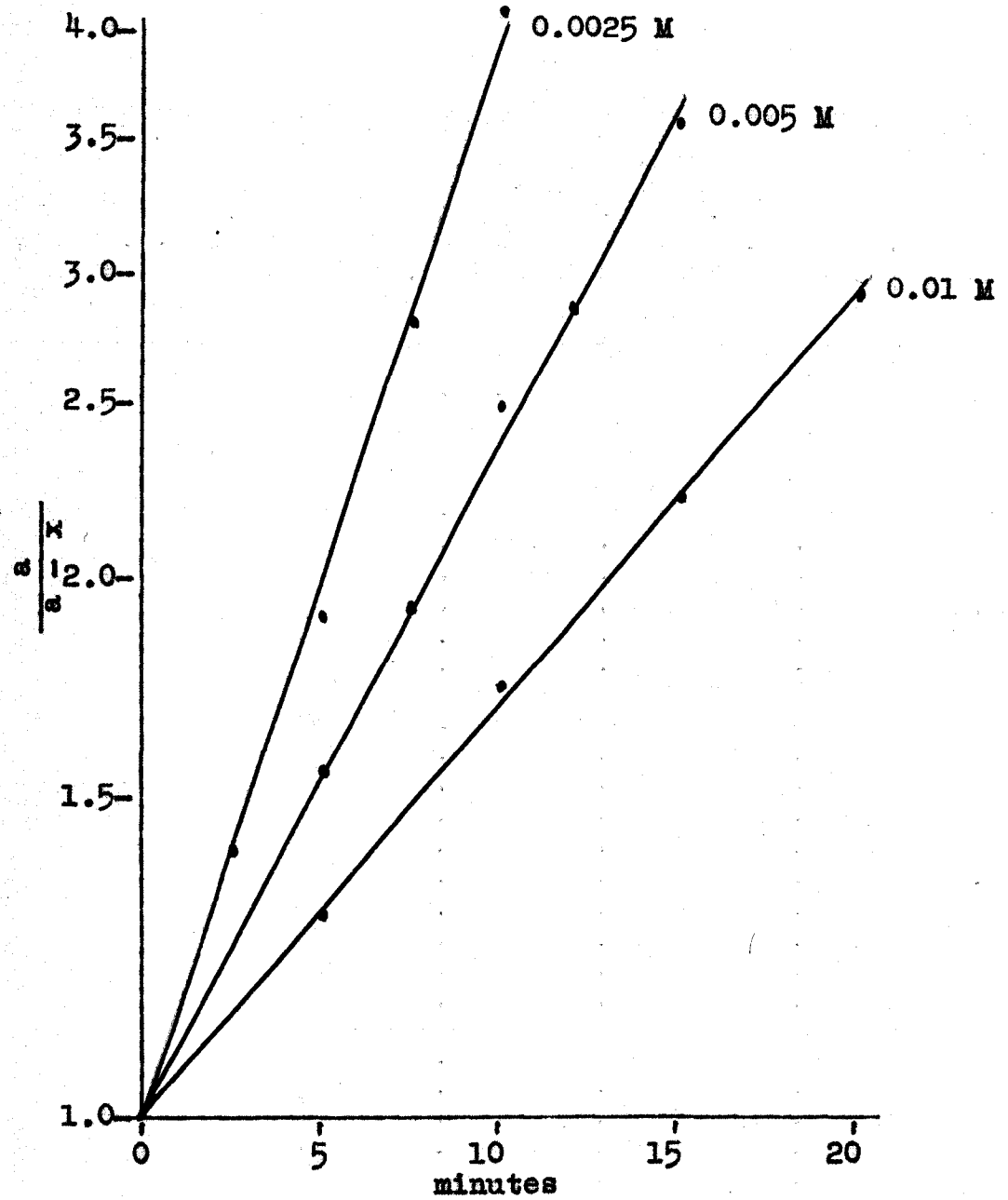

Figure 4. The hydrolysis of AME by Cysteine-Proteinase.

Semilogarithmic plot of $\frac{a}{a - x}$ vs. t

where a = initial substrate concentration in molarity
x = moles hydrolyzed per liter at time, t.

In Tables XIX and XX are summarized the data for the hydrolysis of AME by a later proteinase preparation. It can be seen from Table XIX that enzyme activity, measured by the first order reaction rate constant, is proportional to enzyme concentration. This is shown simply by the fact that the proteolytic coefficient, C, is constant. It will be noted, however, that the proteolytic coefficient, C, at 0.005 M initial substrate concentration, is 2.5 times as large as the proteolytic coefficient obtained from the proteinase preparation in Tables XVII and XVIII at the same initial substrate concentration. This indicates that the proteinase preparation in Table XIX is 2.5 times purer than the proteinase preparation in Tables XVII and XVIII, assuming maximum activation in each case. This can be seen from a consideration of the significance of the proteolytic coefficient. Consider two proteinase preparations, the first twice as pure as the second. If the hydrolyses of AME by these preparations at the same enzyme preparation concentration (mg. of nitrogen/ml. test solution) are compared, the first order constant for the first preparation will be twice that for the second. But the enzyme preparation concentrations in the reaction mixtures are the same in each case, therefore the proteolytic coefficient, K/e, for the first preparation will be twice that for the second. Thus, the proteolytic coefficient is a measure of the relative purity

of proteinase preparations but only when the proteolytic coefficients are compared under the same conditions. The order of the reaction must not change as the purity of enzyme preparations varies.

Table XX shows the effect of initial substrate concentration on the hydrolysis of AME by proteinase. The data are plotted in Figure 5. In this figure the data are plotted according to zero order kinetics, that is, moles per liter hydrolyzed vs. time. This is done in order to visualize the effect of increasing initial substrate concentration on the initial reaction velocity. It can be seen that as initial substrate concentration increases the initial reaction velocity increases and approaches a constant value. This maximum reaction velocity is reached approximately at 0.04 M initial substrate concentration. Figure 5 inset shows that activity is proportional to enzyme concentration.

At first glance it would appear that these reaction kinetics fit the Michaelis-Menten theory of enzyme reaction kinetics (10). However, it will be shown later than one of the products of the reaction, namely arginine, inhibits the reaction and interpretation of the above results by the simple Michaelis-Menten theory is invalid.

Table XIX

$$K = 1/t \log \frac{100}{100 - \%HOH}$$

C is the proteolytic coefficient, = K/e

%HOH = percentage hydrolysis

Table XIX

Effect of Enzyme Concentration on the Cysteine-Proteinase-AME System

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Initial substrate concentration was 0.005 M. Cysteine concentration was 1 mg./ml. of test solution

e	Time	%T	O. D.	%HOH	K	C
	min.					
0.004	0	36	0.444	--	---	---
	5	41	0.387	13		
	10	48	0.319	28	zero	
	15	56	0.252	43	order	
	20	62	0.208	53		
	25	68	0.168	62	0.017	
	30	73	0.137	69	0.017	(4.2)
	35	77	0.114	74	0.017	
	40	81	0.092	79	0.018	
0.006	0	42	0.377	--	---	---
	5	51	0.292	23	0.022	
	10	61	0.215	43	0.024	
	15	70	0.155	59	0.026	
	20	75	0.125	67	0.024	3.8
	25	79	0.102	73	0.023	
	30	83	0.081	79	0.023	
0.008	0	36	0.444	--	---	---
	5	48	0.319	28	0.028	
	10	62	0.208	53	0.033	
	15	69	0.161	64	0.030	4.0
	20	79	0.102	77	0.032	
	25	86	0.066	85	0.033	
0.012	0	41	0.387	--	---	---
	5	60	0.222	43	0.048	
	7	66	0.181	53	0.047	
	10	76	0.119	69	0.051	4.0
	15	85	0.071	82	0.049	
0.016	0	41	0.387	--	---	---
	3	56	0.252	35	0.063	
	5	67	0.174	55	0.070	4.1
	7	72	0.143	63	0.061	

Table XX

Effect of Initial Substrate Concentration on the Cysteine-Proteinase-AME System

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Enzyme concentration was 0.008 mg. of nitrogen/ml. of test solution. Cysteine concentration was 1 mg./ml. of test solution

a	Time	%T	O. D.	x	K	C
	min.					
0.0025	0	55	0.260	---	---	---
	5	68	0.168	0.0009	0.038	
	10	80	0.097	0.0017	0.043	5.1
	15	89	0.051	0.0020	0.047	
	20	92	0.036	0.0022	0.042	
0.005	0	36	0.444	---	---	---
	5	48	0.319	0.0014	0.028	
	10	62	0.208	0.0026	0.033	
	15	69	0.161	0.0032	0.030	4.0
	20	79	0.102	0.0038	0.032	
	25	86	0.066	0.0042	0.033	
0.0075	0	23	0.638	---	---	---
	5	34	0.469	0.0019	0.026	
	10	48	0.319	0.0037	0.030	
	15	58	0.237	0.0047	0.029	3.5
	20	70	0.155	0.0057	0.031	
	30	83	0.081	0.0065	0.030	
	40	88	0.056	0.0068	0.026	
0.01	0	15	0.824	---	---	---
	5	22	0.658	0.0020	0.020	
	10	30	0.523	0.0037	0.020	
	15	41	0.387	0.0053	0.022	
	20	48	0.319	0.0061	0.020	2.8
	30	68	0.168	0.0080	0.023	
	40	78	0.108	0.0087	0.022	
	50	87	0.061	0.0093	0.023	

$$K = 1/t \log \frac{a}{a - x}$$

Table XX (contd.)

a	Time	%T	O. D.	x	K	C
	min.					
0.02	0	13	0.886	---	---	---
	5	17	0.770	0.0026	0.012	
	10	20	0.699	0.0042	0.010	
	15	25	0.602	0.0064	0.011	
	20	29	0.538	0.0078	0.010	1.3
	25	35	0.456	0.0098	0.012	
	40	48	0.319	0.0128	0.011	
	60	62	0.208	0.0154	0.011	
0.04	0	19	0.721	---	---	---
	10	23	0.638	0.0048	0.006	
	20	27	0.569	0.0084	0.005	
	30	31	0.509	0.0116	0.005	0.63
	40	35	0.456	0.0148	0.005	
	50	40	0.398	0.0180	0.005	

$$K = 1/t \log \frac{a}{a - x}$$

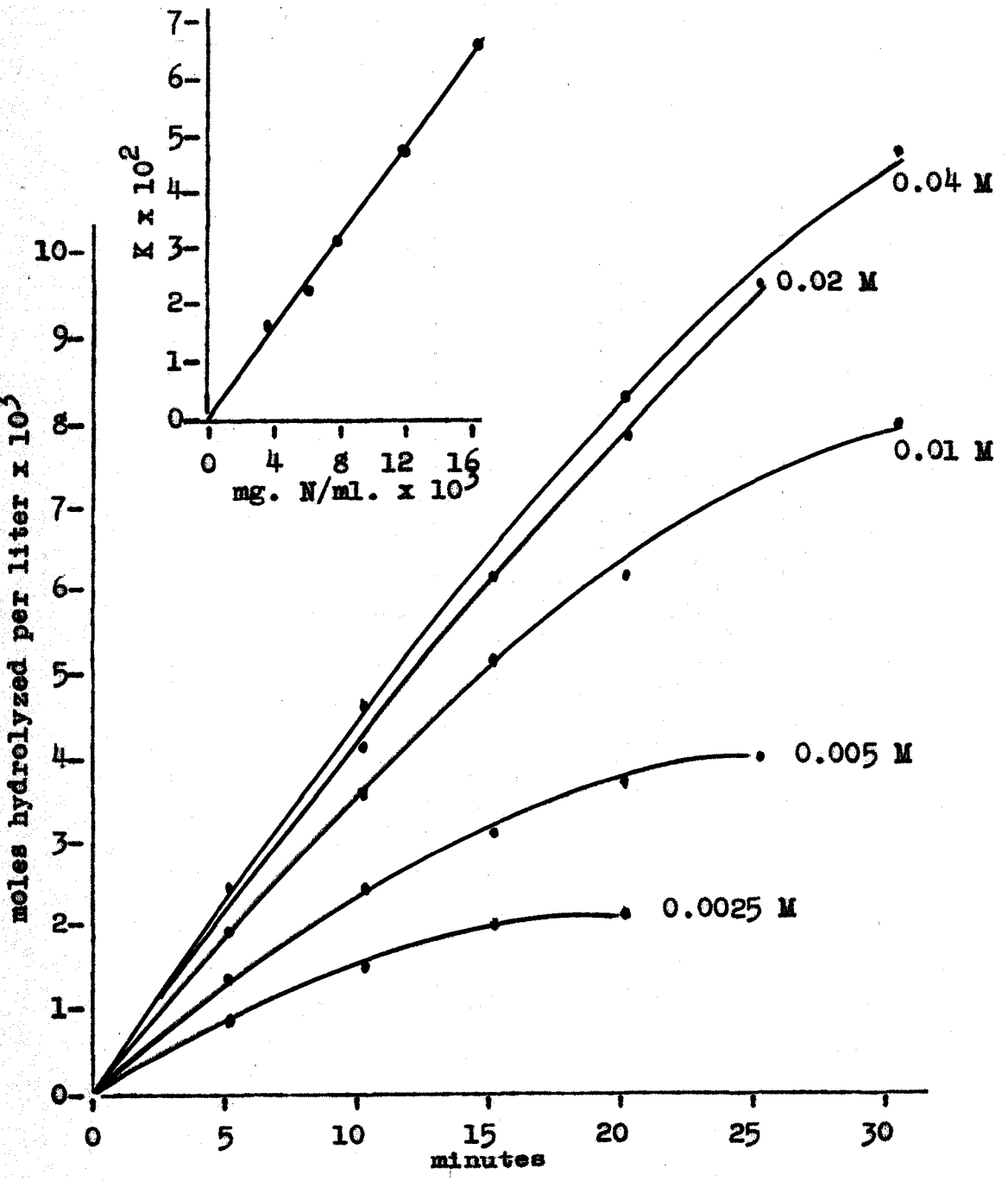

Figure 5. Hydrolysis of AME by Cysteine-Proteinase

Inhibition of the Cysteine-Proteinase-AME System by Arginine. Deviations from simple zero order reaction kinetics are often caused by inhibition of the reaction by one or more of the reaction products. It was of interest to determine whether arginine inhibited the hydrolysis of AME by activated proteinase. Solutions were made up as follows:

Stock Enzyme Solution:-

0.6 ml. of proteinase preparation (1.6 mg.N/ml.) and 100 mg. of cysteine were diluted to 50 ml. with 0.1 M phosphate buffer, pH 7.2.

Stock Substrate Solutions:-

0.02 M and 0.03 M AME solutions, in buffer.

Stock Inhibitor Solution:-

0.08 M solution of arginine in buffer.

Substrate-Inhibitor Solutions:-

a) 10 ml. of 0.08 M arginine + 10 ml. desired AME solution.

b) 5 ml. arginine solution., 5 ml. buffer, 10 ml. AME solution.

c) 5 ml. arginine solution, 15 ml. buffer, 20 ml. AME solution.

d) 2.5 ml. arginine solution, 17.5 ml. buffer, 20 ml. AME solution.

Test Solutions:

	Concn. of Inhibitor
5 ml. AME + 5 ml. buffer + 10 ml. enzyme solution	0.000 M
10 ml. a) + 10 ml. enzyme solution	0.02 M
10 ml. b) + 10 ml. enzyme solution	0.01 M
10 ml. c) + 10 ml. enzyme solution	0.005 M
5 ml. d) + 5 ml. enzyme solution	0.0025 M

The results are tabulated in Table XXI. It can be seen that there was some inhibition at 0.0025 M and 0.005 M inhibitor concentration and considerable inhibition at 0.01 M and 0.02 M inhibitor concentration.

Table XXI

Inhibition of Proteinase-AME System by Arginine

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Initial substrate concentration was 0.005 M. Enzyme concentration was 0.010 mg. nitrogen/ml. of test solution. Concentrations of inhibitor, I, indicated.

I	Time	%T	x
M	min.		
0	0	36	---
	2.5	41	0.0006
	5	44	0.001
	7.5	50	0.0016
	10	53	0.0019
	15	60	0.0025
0.0025	0	36	---
	5	43	0.0009
	7.5	47	0.0013
	10	51	0.0017
0.005	0	36	---
	2.5	38	---
	5	42	0.0008
	7.5	45	0.0011
0.01	0	36	---
	5	41	0.0006
	10	46	0.0012
	15	52	0.0018
0.02	0	36	---
	5	38	---
	10	43	0.001
	15	50	0.0016

The Hydrolysis of BAEE by Cysteine Activated Proteinase. The data for the hydrolysis of BAEE by cysteine-proteinase are shown in Tables XXII and XXIII. The data are plotted in Figure 6. The test solutions and controls were prepared in the same manner as described for AME on page 55. No spontaneous hydrolysis of BAEE was observed.

It can be seen from the Tables and Curve that the hydrolysis of BAEE was zero order over a large portion of the reaction. The zero order constant was independent of initial substrate concentration as it should be. Activity was proportional to enzyme concentration.

The Hydrolysis of BAA by Cysteine Activated Proteinase. The hydrolysis of BAA by cysteine-proteinase is shown in Table XXIV. It can be seen that in the initial portion of the reaction the hydrolysis is zero order and independent of initial substrate concentration. Deviations from zero order kinetics occur much earlier in the reaction than they do for BAEE. Such deviations from zero order kinetics can be explained by inhibition by a reaction product. However, it will be noted that initial reaction velocities for both BAEE and BAA at the same enzyme concentration are identical.

It will be noted that BAEE and BAA are hydrolyzed at about one tenth the enzyme concentration required to

hydrolyze AME within similar reaction times. This is interpreted to mean that the proteinase has a much higher affinity for BAEE and BAA than it has for AME. The occurrence of zero order kinetics for BAEE and BAA and first order kinetics for AME might also indicate a higher affinity for the former substrates.

Table XXII

Effect of Enzyme Concentration on Cysteine-Proteinase-BAEE System

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Initial substrate concentration was 0.005 M. Cysteine concentration was 1 mg./ml. of test solution

e	Time	%T	O. D.	x	K x 10^4	K/e
	min.					
0.0003	0	25	0.602	---	---	---
	5	31	0.509	0.0007	1.4	
	10	39	0.409	0.0016	1.6	
	15	44	0.357	0.0021	1.4	0.47
	20	54	0.268	0.0028	1.4	
	25	64	0.194	0.0034	1.3	
	30	78	0.108	0.0041	1.4	
	35	92	0.036	0.0047		
0.0006	0	25	0.602	---	---	---
	5	35	0.456	0.0012	2.4	
	10	56	0.252	0.0024	2.4	0.42
	15	76	0.119	0.0040	2.7	
	20	96	0.018			
0.0009	0	25	0.602	---	---	---
	2.5	33	0.482	0.001	4.0	
	5	44	0.357	0.002	4.0	
	7.5	59	0.229	0.0031	4.1	0.44
	10	81	0.092	0.0042	4.2	
0.0012	0	25	0.602	---	---	---
	2.5	36	0.444	0.0013	5.2	
	5	56	0.252	0.0024	4.8	0.44
	7.5	78	0.108	0.0041	5.5	

e = enzyme concentration in mg.N/ml. test solution.
x = moles hydrolyzed/liter
K = zero order reaction rate constant; moles per liter hydrolyzed per minute.

Table XXIII

Effect of Initial Substrate Concentration on Cysteine-Proteinase-BAEE System

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Enzyme concentration was 0.0009 mg. nitrogen/ml. of test solution. Cysteine concentration was 1 mg./ml. of test solution

a	Time	%T	O. D.	x	K x 10^4
	min.				
0.005	0		See Table XXII		4.0
0.01	0	5	1.301	---	---
	5	9	1.046	0.002	4.0
	10	16	0.796	0.0039	3.9
	15	29	0.538	0.0059	3.9
	25	84	0.076	0.0094	
0.02	0	6	1.222	---	---
	10	11	0.959	0.0043	4.3
	15	14	0.854	0.006	4.0
	20	18	0.745	0.0078	3.9
	25	24	0.620	0.0098	3.9
	35	38	0.420	0.013	
0.04	0	4	1.398	---	---
	20	8	1.097	0.0086	4.3
	35	14	0.854	0.014	4.0
	45	17	0.770	0.018	4.0
	60	22	0.658	0.022	3.7
	80	32	0.495	0.026	
	100	53	0.276	0.032	
	120	81	0.092		

K = zero order reaction rate constant; moles per liter hydrolyzed per minute.

Table XXIV

The Hydrolysis of BAA by Cysteine Activated Proteinase

Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Enzyme concentration was 0.0009 mg. nitrogen per ml. of test solution. Cysteine concentration was 1 mg. per ml. of test solution. One ml. lf test solution "Conwayed" in each case. Nitrogen obtained from curve, page 17.

a	Time	%T	O. D.	x	K x 10^4
	min.				
0.004	2.5	91	0.041	0.001	4.0
	5	84	0.076	0.002	4.0
	7.5	78	0.108	0.003	4.0
	10	73	0.137	0.0034	
0.008	2.5	91	0.041	0.001	4.0
	5	82	0.086	0.002	4.0
	7.5	78	0.108	0.003	4.0
	10	72	0.143	0.0034	3.4
	12.5	68	0.168	0.0041	
	15	62	0.208	0.005	
	20	55	0.260	0.0063	
	30	50	0.301	0.0072	
0.016	2.5	90	0.046	0.001	4.0
	5	83	0.081	0.002	4.0
	7.5	77	0.114	0.003	4.0
	10	73	0.137	0.0034	3.4
	15	59	0.229	0.0055	3.7
	20	53	0.276	0.0066	
	30	37	0.432	0.010	

K = moles hydrolyzed per liter per minute.

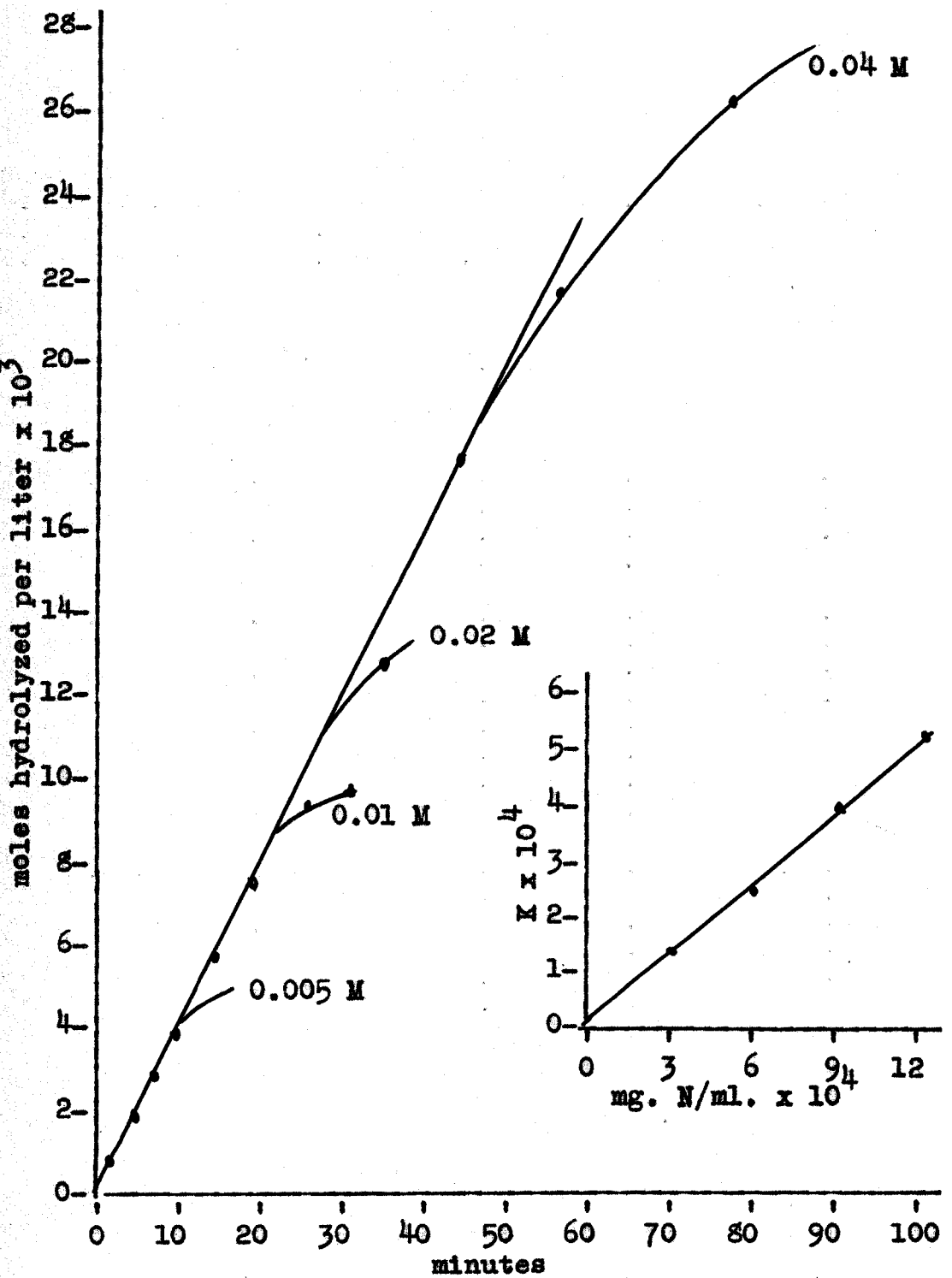

Figure 6. Hydrolysis of BAEE by Cysteine-Proteinase

Determination of the Nature of the Enzymatic Reactions. It was desirable to determine whether there were any other enzymatic reactions occurring besides those involving the ester or amide groups. When AME or BAEE was used as the substrate there was no observation of any NH_3 which could arise from the guanido groups of AME and BAEE or from the alpha amino group of AME. For further verification, chromatograms were run on the AME and BAEE systems after hydrolysis was complete. Tracings of the chromatograms are shown in the following figures. The solvent systems are given on the chromatograms. Figure 7 represents the chromatogram of the proteinase-AME reaction mixture together with arginine, AME, cysteine, and cystine. It can be seen that the reaction mixture gave spots identical to those of arginine, cysteine, and cystine. The spots were obtained by spraying with ninhydrin and drying at 150° C. Figure 8 represents the chromatogram for the proteinase-BAEE reaction mixture together with arginine, benzoylarginine, BAEE. It can be seen that the reaction mixture gave one spot identical with benzoylarginine. The reaction mixture spot and spots from BAEE and benzoylarginine were observed by ultra-violet light (Mineralite-long wave). The arginine spot was then obtained with ninhydrin. The chromatogram shows that the benzoyl group was not hydrolyzed off when BAEE was used as the substrate.

As a final characterization of the proteinase-BAEE system, benzoylarginine was isolated from the reaction mixture by concentration in vacuo, addition of NH_4OH until the oder of NH_3 persisted and cooling in the cold room. The benzoylarginine crystallized out upon cooling, was filtered off, washed with ice cold water and recrystallized from hot water; NH_4OH was added during cooling, m. p. 273° C. (lit. 273° C.). Some of the crystalline product was heated with NaOH, the solution was then acidified and extracted with ether. Upon evaporation of the ether, benzoic acid crystallized, m. p. 121° C.

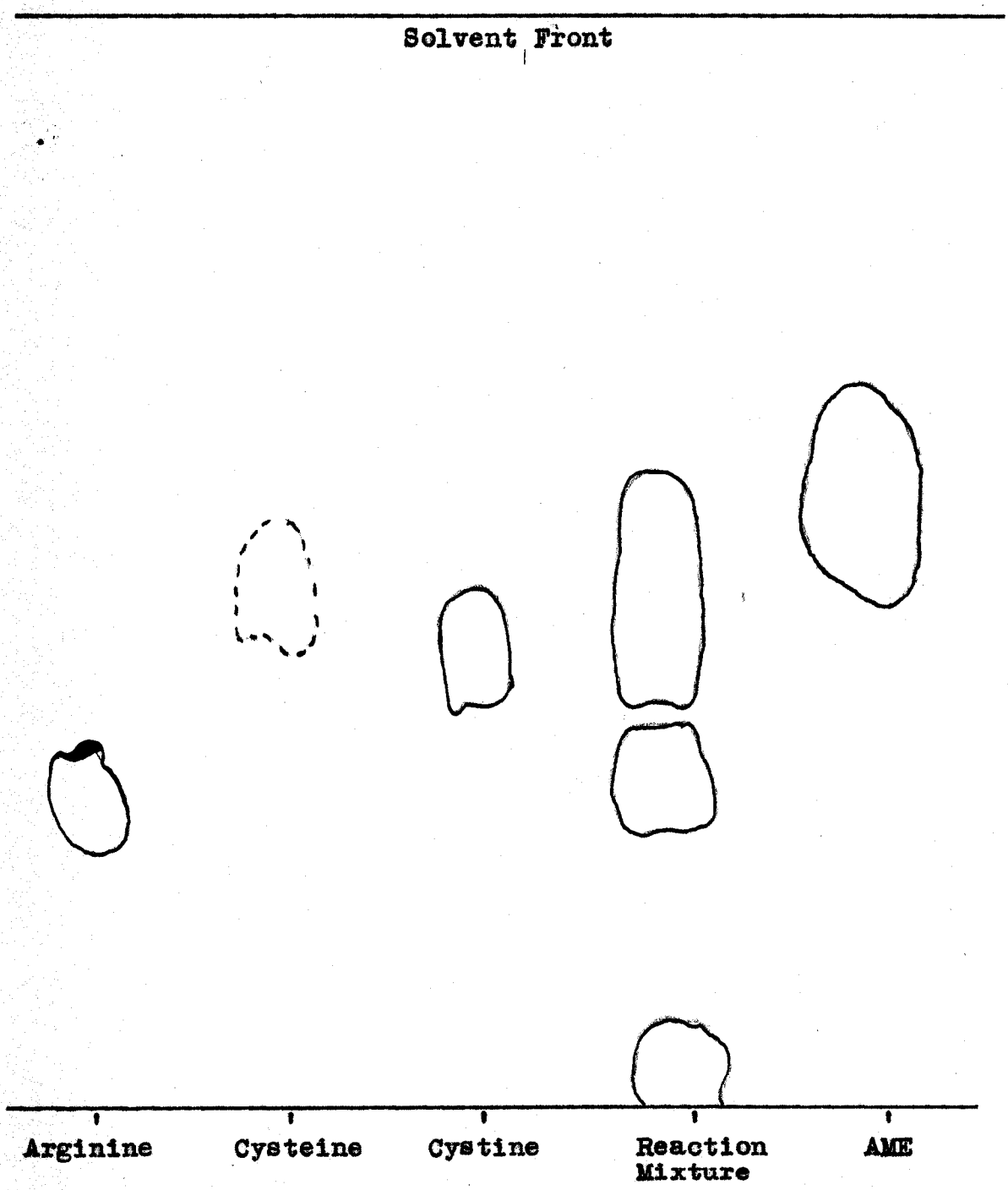

Figure 7. Chromatogram of the Cysteine–Proteinase–AME System
Solvent: methanol 85%, water 11%, triethylamine 4%.

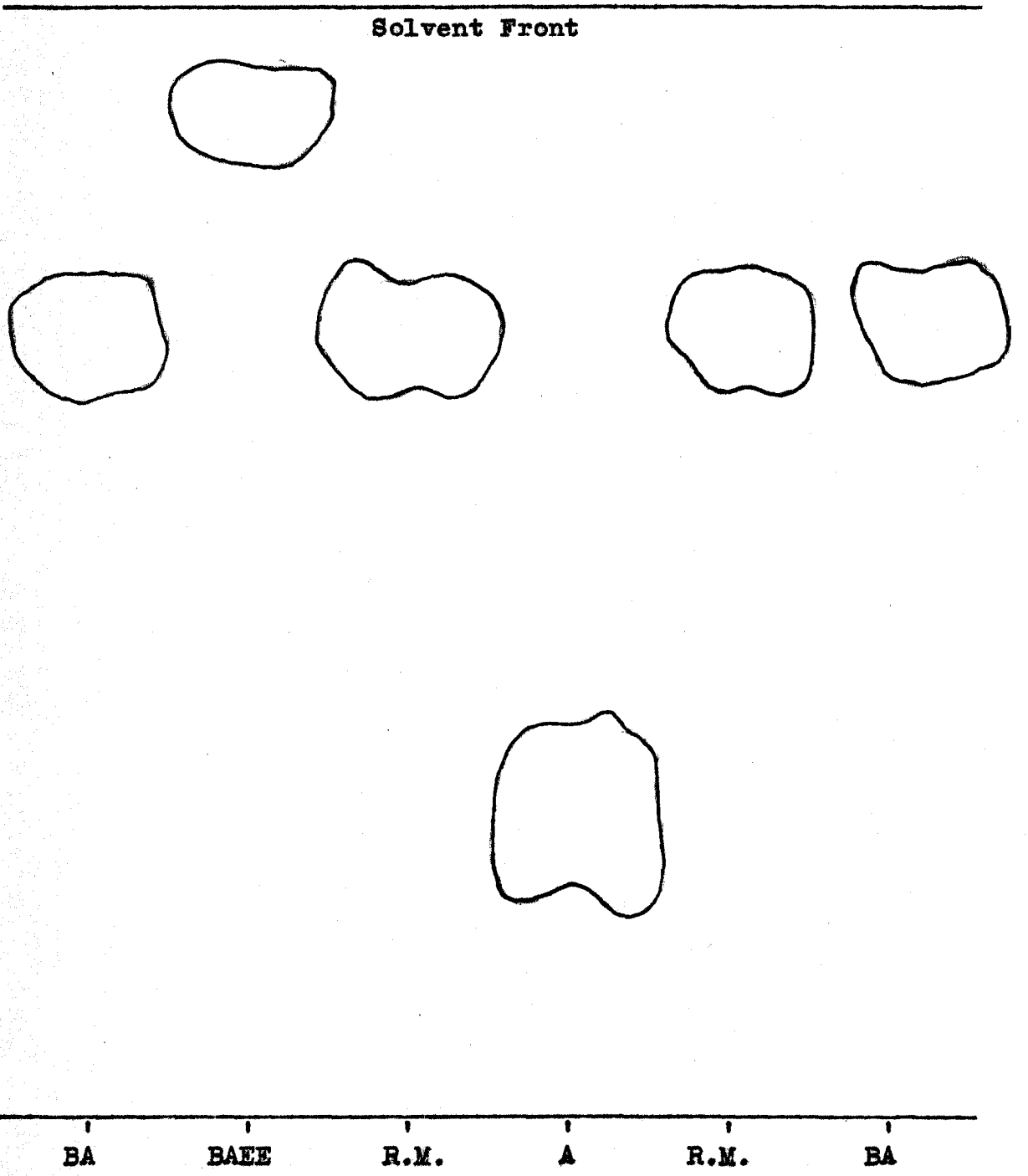

Figure 8. Chromatogram of the Cysteine-Proteinase-BAEE System

BA = benzoylarginine; BAEE = α-benzoylarginine ethyl ester; R. M. = reaction mixture; A = arginine

Solvent: absolute ethanol 80%, water 16%, triethylamine 4%

The Effect of pH on the Cysteine-Proteinase-AME System. It has been reported (2) that the pH optimum for the hydrolysis of gelatin by proteinase is 7. It was of interest to determine the pH optimum for the proteinase-AME system. The data are tabulated in Table XXV. It can be seen that at pH 5.4 there is no hydrolysis after 60 minutes. At pH 6 there is but slight hydrolysis. At pH 7.2 the activity is greatest and the activity decreases at pH 8. Thus the pH optimum is around 7.2.

Activation of the Proteinase. Up to this point the proteinase has been activated either with $FeSO_4$ plus cysteine or with cysteine alone. Table XXVI shows the results of using other activators. It can be seen that cysteine gave the maximum activation under the conditions employed. Perhaps the other substances would give maximum activation in different amounts.

Table XXV

Effect of pH on Cysteine-Proteinase-AME System

Reactions carried out at 37° C. in 0.1 M phosphate buffer at pH indicated. Cysteine concentration was 1 mg./ml. of test solution. Enzyme concentration was 0.0125 mg. N/ml. test solution.

	---a = 0.005 M*----			----a = 0.01 M*----		
pH	Time	%T	O. D.	Time	%T	O. D.
	min.			min.		
5.4	0	47	0.328	0	34	0.469
	5	47	0.328	5	34	0.469
	10	45	0.347	10	34	0.469
	20	45	0.347	20	34	0.469
	60	47	0.328	30	35	0.456
6.0	0	49	0.310	0	34	0.469
	5	49	0.310	5	34	0.469
	10	50	0.301	10	36	0.444
	15	51	0.292	20	37	0.432
	20	51	0.292	30	41	0.387
	60	63	0.201			
7.2	0	49	0.310	0	34	0.469
	5	52	0.284	5	38	0.420
	10	58	0.237	10	42	0.377
	20	71	0.149	20	49	0.310
	30	79	0.102	30	54	0.268
	45	85	0.071			
	60	92	0.036			
8.0	0	48	0.319	0	30	0.523
	5	50	0.301	5	32	0.495
	10	56	0.252	10	36	0.444
	20	60	0.222	20	42	0.377
	30	71	0.149	30	47	0.328
	45	77	0.114			
	60	84	0.076			

a = initial substrate concentration
* Different enzyme preparations

Table XXVI

Activation of the Proteinase

Reactions carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2. Initial substrate concentration was 0.005 M. Enzyme concentration was 0.012 mg. N/ml. of test solution. Concentration of activators was 0.008 M in each case.

Activator	Time	%T	O. D.
	min.		
Cysteine	0	39	0.409
	10	59	0.229
	15	70	0.155
	20	81	0.092
	30	90	0.046
Glutathione	0	39	0.409
	10	55	0.260
	20	71	0.149
	30	83	0.081
	45	91	0.041
KCN	0	38	0.420
	10	39	0.409
	15	45	0.347
	20	48	0.319
	30	61	0.215
	45	72	0.143
	60	82	0.086
	80	90	0.046

Modifications in the Method of Preparing the Proteinase. Several modifications in the method of preparation of the proteinase were tried in an attempt to obtain a purer enzyme preparation. The activities of these preparations were compared by comparing the proteolytic coefficients, C, of the hydrolysis of AME at 0.005 M initial substrate concentration, 37° C., 0.1 M phosphate buffer, pH 7.2.

1. Effect of Varying the pH of the Cell-free Filtrate Before Precipitation with Methanol. The pH of all the cell-free filtrates from which the proteinase was precipitated was around 7. It was of interest to determine whether the proteinase could be precipitated undenatured at other pH values. The pH of the cell-free filtrate was adjusted from 4.1 to 9 with acetic acid or NaOH respectively and then precipitated in the usual manner with methanol at 67% final concentration. The results from four different cell-free filtrates are shown in Table XXVII. It can be seen that the highest activity was obtained at pH 6.0 to 6.6. It is interesting to note that at the lowest pH values, namely pH 4.1 to 4.3, the enzyme precipitated out in 15 to 30 minutes and could be harvested at that time. Above pH 4.3 the enzyme required longer times to precipitate and at pH 7 it was 24 to 48 hours before precipitation was complete. This observation indicates that the isoelectric point of the proteinase might be near pH 4. Preliminary electrophoretic

studies on paper have been unsuccessful in verifying this because at no pH was it possible to obtain any movement of the proteinase. This was probably due to strong absorption of the enzyme to the paper. The proteinase preparations obtained from cell-free filtrates adjusted to pH 4.1 to 5.5 were highly pigmented. From pH 6.0 to 8.0 the preparations were nearly colorless. The presence of the pigment, dark green in color, might account for the lower activity of the preparations obtained at the lower pH values. The pigment could be dialyzed out of these preparations but the activity was not increased appreciably by so doing.

2. Effect of Varying the Concentration of Methanol Used to Precipitate the Proteinase. In the above study of the effect of pH on the precipitation and activity of the proteinase, it was observed that at the lower pH values the precipitate started forming before the total amount of methanol was added. Therefore an experiment was done in which the concentration of methanol was varied from 40% to 67%. The experiment had to be done at pH 5 because no precipitate could be obtained with these low concentrations of methanol when the cell-free filtrate was at a pH higher than 5. The results are shown in Table XXVIII. It can be seen that the highest activity was obtained at 45% to 55% methanol. These preparations were also highly pigmented.

If the cell-free filtrate was adjusted to pH 5, precipitated with 40% methanol and the precipitate removed, then the supernatant yielded more precipitate upon increasing the methanol concentration to 55%. The preparation from this 55% portion was no more active than one initially precipitated at 55% methanol.

Table XXVII

Effect of Varying pH of Cell-free Filtrate Before Precipitation with Methanol

Results obtained with four different cell-free filtrates.

pH	Proteolytic Coefficient
4.1	2.2
5.1	2.2
5.8	2.2
6.5	3.7
7.0	1.4
4.2	1.6
5.0	1.7
6.0	1.9
7.1	1.2
8.0	1.0
5.8	1.8
6.6	2.3
7.2	1.8
5.5	2.6
6.6	3.8
7.2	3.1
9.0	1.0

Table XXVIII

Effect of Varying the Concentration of Methanol Used to Precipitate the Proteinase

Final Volume Percentage of Methanol	Proteolytic Coefficient
40	2.0
45	2.3
50	2.4
55	2.3
60	1.8
67	1.5

3. Acetone Precipitation. In order to compare the methanol precipitated proteinase with the acetone precipitated enzyme of Kocholaty (4), portions of a cell-free filtrate, unadjusted, were precipitated with 50% acetone according to the method of Kocholaty and with 67% methanol under the same conditions. The resulting preparations had identical activity on AME.

4. Precipitation of the Proteinase in the Presence of Zinc Acetate with Low Concentrations of Ethanol. High concentrations of alcohol or acetone can denature enzymes especially when they are in contact with the enzyme over a long period of time. Cohn (7) has devised a method for precipitating blood proteins in the presence of zinc salts which requires much lower concentrations of alcohol. The

proteinase was prepared in the following manner: To 200 ml. of the cell-free filtrate adjusted to pH 5.6 were added 400 ml. of methanol and the precipitate was harvested in the usual manner. The precipitate was extracted with 15 ml. of 1% NaCl, centrifuged and the insoluble residue discarded. To the clear supernatant were added 5 ml. of 0.1 M acetate buffer, pH 5.5, and 0.15 ml. of 0.025 M zinc acetate buffer, pH 5.6. After cooling to -5° C., 4 ml. of ethanol (pre-cooled) were added. The resulting precipitate after centrifugation and decantation was extracted with 20 ml. of 0.1 M phosphate buffer, pH 7.2, centrifuged and the insoluble residue discarded. Thus the activity of the clear supernatant could be determined in phosphate buffer. The proteolytic coefficient of this preparation was 8.6 as compared to 1.8 for the proteinase prepared from the same cell-free filtrate in the usual manner. Precipitation with lower concentrations of ethanol (9 to 14%) in the presence of zinc acetate resulted in little or no increase in activity. It is of interest that the proteinase preparations precipitated in the presence of zinc salts had a slight activity toward AME without the addition of cysteine. However, when zinc acetate was added to the usual proteinase preparation in tris-(hydroxymethyl)-aminomethane buffer, no activation

toward AME was obtained. The use of the zinc-ethanol precipitation is now being studied to determine whether it can be applied to the cell-free filtrate and eliminate the previous precipitation with methanol.

The Stability of Cysteine-Proteinase at 37° C.

In order to test the stability of the proteinase under the condition of the reaction, the enzyme plus cysteine was incubated for various intervals at 37° C. The substrate, AME, was then added and samples withdrawn at intervals and assayed for residual ester. The results are shown in Table XXIX. It can be seen that there is no appreciable inactivation of the proteinase after 120 minutes incubation at 37° C. which is about the maximum time used for any enzyme reactions in this research. After 12 hours incubation at 37° C. the proteinase was only slightly inactivated.

Similar heat stability studies at 50° C. were carried out and it was found that the proteinase was slowly inactivated after 30 minutes incubation at 50° C. It was also noted that addition of cysteine to the proteinase preparation before heat treatment resulted in a stabilization of the enzyme toward heating at 50° C.

Table XXIX

Proteinase incubated at 37° C. in 0.1 M phosphate buffer, pH 7.2, in the presence of 2 mg. of cysteine/ml. of solution. After addition of the substrate, enzyme concentration was 0.020 mg. N/ml. of test solution. Initial substrate concentration was 0.005 M. Reactions were carried out at 37° C. in 0.1 M phosphate buffer, pH 7.2.

Time of Incubation at 37° C. Before Addition of AME	After Addition of AME	O. D.	x
min.	min.		
0	0	0.398	---
	5	0.244	0.0019
	10	0.131	0.0034
	15	0.056	0.0043
30	0	0.420	---
	5	0.237	0.0022
	10	0.114	0.0036
	15	0.051	0.0044
60	0	0.387	---
	5	0.208	0.0023
	10	0.137	0.0032
	15	0.056	0.0043
120	0	0.420	---
	5	0.252	0.0020
	10	0.137	0.0034
	15	0.076	0.0041
720	0	0.444	---
	5	0.301	0.0016
	10	0.201	0.0027
	15	0.125	0.0036

x = moles hydrolyzed per liter

SUMMARY AND CONCLUSION

Collagenase preparations, made by the method described, hydrolyze α-benzoyl-L-argininamide and the ethyl, benzyl and isopropyl esters of α-benzoylarginine. When these collagenase preparations are heated at 50° C. for 5 to 30 minutes, the activity toward native collagen is completely destroyed but there remains a small but constant residual activity toward the synthetic substrates. This residual activity is increased by the addition of cysteine. This activation by cysteine is characteristic of a proteinase also secreted by Cl. histolyticum. The proteinase has no activity toward collagen. Therefore it was concluded that the collagenase preparations were contaminated with this proteinase. This does not preclude the possibility that cysteine converts the collagenase to proteinase. Likewise, it cannot be concluded that the collagenase itself hydrolyzed the synthetic substrates.

The collagenase preparations failed to hydrolyze arginine methyl ester. After heating the preparations to destroy collagenase, the heat treated preparations still did not hydrolyze arginine methyl ester. However, addition of cysteine to the original collagenase preparations or to the heat treated preparations resulted in the hydrolysis of this substrate. Here again, the results indicated the presence

of the cysteine activatable proteinase in the collagenase preparations. Again the possibility of a conversion of collagenase to proteinase under these conditions is not precluded, although this seems improbable. All of the collagenase preparations were identical in this respect.

By a different method of preparation it was possible to obtain the cysteine activatable proteinase free from any activity toward collagen. The proteinase, when activated by cysteine, hydrolyzed α-benzoylargininamide, the ethyl, benzyl, and isopropyl esters of α-benzoylarginine, arginine methyl ester and, very slowly, lysine methyl ester. The hydrolysis of the esters and amide of α-benzoylarginine by activated proteinase was zero order over a large part of the reaction. The hydrolysis of arginine methyl ester by activated proteinase was apparent first order, the first order constant decreasing with increasing initial substrate concentration. The pH optimum for the hydrolysis of arginine methyl ester was about 7.2. The proteinase could be activated by cysteine, glutathione and to a lesser extent by KCN.

The proteolytic coefficient for the hydrolysis of arginine methyl ester, under constant conditions of pH, temperature, buffer and initial substrate concentration, can be used for determining the relative purity of different

proteinase preparations. The other substrates mentioned could be used as well for following the purification of the proteinase except that they might also be hydrolyzed by collagenase, should conditions of the preparation of the proteinase favor collagenase preparation also.

Slightly better proteinase preparations are obtained if the pH of the cell-free filtrate is adjusted to about pH 6.6 before precipitation with methanol. A four-fold purification of the proteinase can be obtained by precipitation in the presence of zinc acetate with ethanol (final concentration of 17%).

The proteinase is slowly inactivated by heating at 50° C. for 30 minutes. It is not inactivated after heating at 37° C. for 120 minutes.

PREPARATION OF THE SUBSTRATES

The synthetic substrates were prepared essentially according to the methods referred to after the names of the substrates. The actual procedures are given here because in most cases the methods given in the literature were modified. Some of the preparations are intermediates.

Collagen. Collagen was prepared by the method of Neuman (13). The source was cattle achilles tendon. Adhering membrane, fat, blood, and mucus were removed. The tissues were washed well with water and cut up into small pieces. Several pieces at a time were shredded in a Waring blendor in an ice and water medium. The fibers were removed and washed with distilled water then suspended in 50 times their weight of 10% NaCl solution. The suspension was stored in the cold room at 4° C. for two weeks. During this period the NaCl solution was renewed several times. The NaCl solution was then removed, the fibers washed with distilled water and suspended in M/15 Na_2HPO_4 solution at 4° C. for three days. The Na_2HPO_4 solution was renewed several times during this period. Next the Na_2HPO_4 solution was removed, and the fibers were washed with distilled water until free of inorganic salts. Finally the fibers were successively treated for 20 hours in 20 times their weight of acetone, alcohol, and ether at 4° C. The fibers were then air dried.

Glycine Hydrochloride. One mole (75 g.) of glycine was dissolved in 100 ml. of water in a large evaporating dish and 166 ml. of 1:1 HCl were added with stirring. The solution was evaporated on the steam bath to approximately 50 ml. Upon approaching this volume some of the glycine hydrochloride crystallized out as white needles. The mixture was cooled in the ice chest and then the glycine hydrochloride was filtered off. The filtrate was evaporated to near dryness on the steam bath, cooled and the crystals filtered off as before. The crystals were sucked dry as possible on the Büchner funnel then dried in air. Yield, 105 g.
M. p., 178-182° C. (lit. 185° C.).

The glycine hydrochloride can be washed with ice cold absolute methanol or recrystallized from methanol. This is unnecessary when the material is to be used as an intermediate in a later preparation.

Glycine Ethyl Ester Hydrochloride. In a two liter round bottom short neck flask equipped with a reflux condenser was placed 205 g. (1.8 mole) of glycine hydrochloride and 950 ml. of absolute ethyl alcohol previously saturated with dry HCl gas. The glycine hydrochloride dissolved upon heating and the solution was refluxed for 3-1/2 hours (bumping stones). The solution was then cooled in the cold room and upon seeding or merely pouring the solution from the flask into a beaker, the ester hydrochloride crystallized

out in white fluffy needles. The crystals were filtered off and the filtrate evaporated on the water bath to about one third its volume, then cooled. Another portion of ester salt was obtained and was filtered off. The glycine ethyl ester hydrochloride was dried in air. Yield, 215 g. M. p., 137-141° C. (lit. 144° C.).

The ester hydrochloride can be recrystallized from absolute ethanol but this is unnecessary when the material is to be used in a later synthesis.

Glycine Anhydride (Diketopiperazine) (14). 195 g. of glycine ethyl ester hydrochloride were covered over with 120 ml. of water and cooled to -5° C. Fifty-one grams of NaOH were dissolved in 100 ml. of water, cooled to -5° C. and added slowly to the ester hydrochloride suspension over a period of 1/2 hour with constant stirring. The temperature was not allowed to rise over -5° C. The ester hydrochloride dissolved slowly as the NaOH was added. The solution was allowed to stand for 24 hours at room temperature during which time some glycine anhydride crystallized out. The mixture was placed in the deep freeze until at -5° C. then filtered and pressed dry. The anhydride was washed with small amounts of ice cold water, filtered, pressed dry and dried in air. Yield, 32 g. Chars at 250° C., begins to decompose at 260° C., melts with further decomposition at 320° C. (copper block).

Glycylglycine (14). One gram of finely powdered glycine anhydride was shaken at room temperature with 10 ml. of N NaOH. The anhydride dissolved slowly and after 20 minutes the solution was neutralized with 10 ml. of N HCl. Absolute ethanol was added very slowly to the neutralized solution, and with stirring and scratching the glycylglycine crystallized out. Excess ethanol must be avoided or NaCl precipitates out also. The glycylglycine was recrystallized from aqueous ethanol. M. p., 259° C. (lit. 260° C.).

$C_4H_8N_2O_3$. Calculated, N 21.2; found, N 21.6

Chloroacetylglycylglycine (25). Eighteen grams of finely powdered glycine anhydride were dissolved in 80 ml. of water containing 6.8 g. NaOH. The anhydride dissolved slowly. The solution was poured into a 500 ml. round bottom three-necked flask equipped with an electric stirrer and two dropping funnels, and cooled to 0° C. 20.5 g. of chloroacetyl chloride and 40 ml. of water containing 8 g. of NaOH were added alternately with constant stirring at such a rate that the temperature remained at 0° C. (ice bath). After the addition of the chloroacetyl chloride and NaOH, the solution was acidified dropwise with 18 ml. of conc. HCl diluted to 30 ml. with water. The temperature was not allowed to rise above 10° C. during the acidification. The reaction mixture began to solidify during the acidification.

Upon cooling the entire reaction mixture solidified. The solid was filtered off and pressed dry. The filtrate was placed in the cold room for 24 hours and more product was obtained. The combined product (20 g.) was washed with ice cold water and recrystallized from 70 ml. of hot water (Darco). The product crystallized in white needles. Yield after second recrystallization 10 g. M. p., 175-177° C. (lit. 178° C.).

$C_6H_9ClN_2O_4$. Calculated. N 13.4, Cl 17.0
Found. N 13.4, Cl 17.4

Glycylglycylglycine (24). Seven grams of chloroacetylglycylglycine were dissolved in 35 ml. of concentrated NH_4OH, placed in a pressure bottle, stoppered and let stand at room temperature for 24 hours. The solution was then concentrated to about 5 ml. under reduced pressure and at a temperature of about 60° C. The thick liquid was diluted to 15 ml. with water and 150 ml. of absolute ethanol was added with stirring. An oil settled out which upon stirring and cooling solidified to a white amorphous mass. The product was filtered off and pressed dry and dried in air. The white powder was dissolved in 50 ml. of water and 75 ml. of ethanol were added slowly with stirring. Upon scratching the diglycylglycine crystallized out. The product was filtered, and dried in air. M. p., colors at 205° C., begins to melt at 230° C. (lit. 246° C.).

$C_6H_{11}N_3O_4$. Calculated, N 22.2; found, N 22.4

Glycylglycine Ethyl Ester Hydrochloride (14). Ten grams of very finely pulverized glycine anhydride were suspended in 270 ml. of absolute ethyl alcohol. The suspension was saturated with dry HCl gas with cooling and then warmed quickly in a water bath to boiling. Most of the anhydride dissolved upon heating. The hot mixture was filtered in a hot water funnel and upon cooling in the cold room overnight the ester hydrochloride crystallized out in plates. The crystals were filtered off, washed with ether and dried in air. M. p., 182° C. (lit. 182° C.).

$C_6H_{13}ClN_2O_3$. Calculated, N 14.2; found, N 14.2

Benzoylglycyl Chloride (Hippuryl Chloride) (23). Eight grams of finely powdered benzoylglycine (hippuric acid) were placed in a pressure bottle, 70 g. of acetyl chloride were added followed by 10 g. of phosphorus pentachloride. The bottle was sealed and shaken on a mechanical shaker for 3 hours. The benzoylglycine slowly dissolved in the acetyl chloride and the benzoylglycyl chloride crystallized out as it formed. Thus the suspension of the amorphous hippuric acid in the reaction mixture gradually changed over into a crystalline mass. The reaction mixture was filtered very rapidly and the crystals washed four times with

petroleum ether. The material was dried in a vacuum desiccator over P_2O_5. The hippuryl chloride was a light yellow crystalline mass. It was converted to the amide without previous recrystallization.

Benzoylglycinamide (Hippuramide) (23). About 350 ml. of anhydrous ether were placed in a 500 ml. erlenmeyer flask and saturated with dry NH_3 gas with exclusion of moisture (soda-lime). While the stream of NH_3 was still passing into the ether small amounts of hippuryl chloride were added with vigorous shaking until the entire yield from the above preparation had been added. The NH_3 stream was continued for 15 minutes and the flask was shaken frequently. There was no visible solution of the hippuryl chloride; hippuramide and NH_4Cl replaced the former as the reaction proceeded. The contents of the flask were filtered off and recrystallized from just slightly more than the amount of hot water necessary to dissolve the mixture (Darco). The hippuramide crystallized out as white needles. Yield 60% of theoretical, based on weight of hippuric acid used. M. p., 183-184° C. (lit. 183° C.).

$C_9H_{10}N_2O_2$. Calculated, N 15.7; found, N 15.5

L-Leucine Methyl Ester Hydrochloride ((15) for the D-isomer). Ten g. of L-leucine were covered over with 60 ml. of absolute methanol previously saturated in the cold with dry HCl gas. The mixture was again saturated with dry HCl

gas with cooling. The solution was stoppered and let stand at room temperature for one day. The alcohol and HCl were evaporated off in vacuo and the residue was taken up in absolute methanol. Anhydrous ether was added to precipitate the ester salt. The leucine methyl ester hydrochloride was recrystallized from methanol-ether. Yield, 11 g. M. p., 150° C. (lit. 150° C.).

$C_7H_{16}ClNO_2$. Calculated, N, 7.2; found, N 7.1

L-Leucinamide Hydrochloride (16). Ten grams of L-leucine methyl ester hydrochloride were covered over with 50 ml. of anhydrous methanol previously saturated with dry NH_3 at 0° C. The solution was allowed to stand at room temperature for 2 days. The solution was then repeatedly concentrated in vacuo with methanol to remove all of the NH_3. Upon removal of the methanol in vacuo the amide hydrochloride crystallized out. It was washed with ether and recrystallized from methanol-ether. M. p., 236-237° C. (lit. 236-237° C.).

$C_6H_{15}ClN_2O$. Calculated, Cl 22.7; found, Cl 22.9

$$[\alpha]_D^{25} = +10.0 \text{ (5\% in } H_2O)$$

reported: $$[\alpha]_D^{25} = +9.5 \text{ (5\% in } H_2O)$$

L-Proline Benzyl Ester Hydrochloride (17). Ten grams of L-proline were dissolved in 100 ml. of benzyl alcohol previously saturated with dry HCl gas. The solution was gassed again with dry HCl without cooling, stoppered, and let stand at room temperature overnight. The solution was again gassed with dry HCl and let stand at room temperature for 2 days. The HCl and H_2O were distilled off *in vacuo* at a temperature not higher than 80° C. The solution was then cooled, poured into a beaker and 500 ml. of ether added. Upon scratching and cooling in deep freeze the ester salt precipitated. It was washed with ether and dried in a vacuum desiccator over $CaCl_2$. All of the benzyl alcohol could not be removed. Yield, 16 g. M. p., 121-136° C. lit. 148° C.). A product with a sharper melting point could not be obtained.

L-Prolinamide Hydrochloride (17). Attempts to prepare prolinamide hydrochloride by ammonolysis of the ester hydrochloride yielded a product which melted above 250° C. Therefore the free ester was prepared from the ester salt and ammonolysis of the former undertaken. 7.5 g. of proline benzyl ester hydrochloride were covered over with 5 ml. of water and 25 ml. of ethyl acetate added. 1.4 g. of NaOH in 4 ml. of water were added slowly with shaking and external cooling. Anhydrous K_2CO_3 was added until a thick suspension was obtained. The suspension was shaken vigorously for

5 minutes and the ethyl acetate solution was poured into a clean flask and stoppered. The K_2CO_3 paste was extracted twice more with 20 ml. portions of ethyl acetate. The ethyl acetate solutions were poured together and shaken for 10 minutes with fresh anhydrous K_2CO_3. The ethyl acetate solution was poured into a clean flask and dried over anhydrous Na_2SO_4 for 2 hours. The solution was then poured into a distilling flask and 3 times its volume of absolute methanol added. The solution was evaporated in vacuo at 35° C. to its original volume. This was repeated twice, the last time the solution was distilled until no more distillate came over at 35° C. The residual volume was about 5 ml. This was poured into a pressure bottle, 75 ml. of absolute methanol previously saturated with dry NH_3 at 0° C. were added, the bottle was sealed and let stand at room temperature for 3 days. A slight precipitate formed and was filtered off. The filtrate was evaporated twice in vacuo at 35° C. with methanol to remove NH_3. Upon removal of the methanol in vacuo at 35° C., the free amide precipitated out. It was filtered, washed with ether and dried in air. Yield, 1 g. M. p., 81-90° C. The free amide was dissolved in a little methanol and ether was added until the amide nearly precipitated. Dry HCl gas was passed into the solution and the amide hydrochloride precipitated out. It was recrystallized from methanol-ether. M. p., 174-176° C.

$C_5H_{11}ClN_2O$. Calculated, N 18.6; found, N 18.2

L-$C_5H_{11}ClN_2O$. Calculated, N 18.6; found, N 18.2

of L-arginine monohydrochloride were dissolved in 100 ml. of absolute methanol previously saturated in the cold with dry HCl gas. The solution was gassed again with dry HCl without cooling, stoppered and let stand at room temperature for 24 hours. The solution was then concentrated to about 50 ml. under reduced pressure and at a temperature not over 40° C. The residue was poured into a beaker and commercial ether was added to precipitate the ester salt. The arginine methyl ester dihydrochloride was recrystallized by dissolving in methanol and adding ether slowly nearly to turbidity. Upon scratching and cooling the ester salt crystallized in prisms. Yield, 5 g. M. p., 193° C. with decomposition (lit. 193° C. with decomposition).

$C_7H_{18}Cl_2N_4O_2$. Calculated. Cl 27.2, N 21.1
Found. Cl 27.3, N 21.1

$$[\alpha]_D^{25} = +21.2 \text{ (5\% in N HCl)}$$

α-Benzoyl-L-Arginine (18). To a solution of 21.2 g. of arginine monohydrochloride in 120 ml. of water were added alternately in four portions 15 ml. of benzoyl chloride and a solution of 17 g. of Na_2CO_3 in 170 ml. of water. The reaction mixture was stirred vigorously and the temperature was kept below 30° C. The reagents were added

at such a rate that the reaction mixture was at all times quite alkaline (light red to phenolphthalein). Addition of the reagents required about 1 hour. After addition of the reagents the reaction mixture was stirred for another hour then acidified dropwise to Congo Red with concentrated HCl. The slight precipitate of benzoic acid and dibenzoylarginine was filtered off and the filtrate was extracted several times with 100 ml. portions of ether to remove dissolved benzoic acid. The aqueous layer was adjusted to pH about 8.5 and concentrated in vacuo at 40° C. to about 100 ml. The residue was poured into a beaker and NH_4OH was added until the odor of NH_3 persisted. The sides of the beaker were scratched and the beaker placed in the cold room at 4° C. overnight. The α-benzoylarginine crystallized out in white prisms. The crystals were filtered off, washed well with small portions of ice cold water and recrystallized from hot water with NH_4OH added during cooling as described above. Yield, 80% of theory. M. p., 273° C. (lit. 273° C.).

$C_{13}H_{18}N_4O_3$. Calculated, N 20.1; found, N 20.1

α-Benzoyl-L-Arginine Benzyl Ester Hydrochloride (19). Two g. of α-benzoylarginine were dissolved in 35 ml. of benzyl alcohol and gassed with dry HCl without cooling. The flask was stoppered and let stand at room temperature for one day, gassed again with HCl and let stand another 24 hours. Ether was added to precipitate the ester salt

as an oil. The oil was washed with several portions of ether. The oil was then dissolved in a small amount of benzyl alcohol and ether was added to turbidity. Upon cooling in the deep freeze the ester hydrochloride crystallized out. It was filtered off, washed with ether and dried in a vacuum desiccator over P_2O_5. A trace of benzyl alcohol remained in the product. M. p., 74-77° C. (lit. 75-78° C.).

$C_{20}H_{25}ClN_4O_3$.	Calculated.	Cl 8.8, N 13.8
	Found.	Cl 8.8, N 13.4

α-Benzoyl-L-Arginine Ethyl Ester Hydrochloride (19). Ten grams of α-benzoylarginine were dissolved in 700 ml. of absolute ethanol previously saturated with dry HCl. The solution was then gassed again with HCl, stoppered, and let stand at 4° C. for 24 hours. The solution was concentrated in vacuo at 35° C. and the residual syrup was re-esterified as above. The solution was again concentrated and ether added to precipitate the ester salt as an oil. The ether was poured off and the oil washed several times with ether. The oil was then covered over with ether, stoppered, and placed in the deep freeze for 3 days during which time the ester hydrochloride solidified. The ester salt was filtered off, redissolved in a small amount of ethanol, and ether was added to turbidity. The mixture was placed in the deep

freeze for 2 days during which time the ester hydrochloride crystallized. The crystals were filtered, washed with ether and dried in a vacuum desiccator over $CaCl_2$. M. p., 134-135° C. (lit. 129-130° C.).

$C_{15}H_{23}ClN_4O_3$. Calculated. Cl 10.3, N 16.3
Found. Cl 10.4, N 16.3

$[\alpha]_D^{25} = -12.0$ (5% in N HCl)

α-Benzoyl-L-arginine Isopropyl Ester Hydrochloride (19). This substrate was prepared in exactly the same manner as the benzyl ester, except that isopropyl alcohol (redistilled) was used throughout instead of the benzyl alcohol. M. p., 173-174° C.

$C_{16}H_{25}ClN_4O_3$. Calculated, Cl 9.9; found Cl 10.0

α-Benzoyl-L-Argininamide Hydrochloride Monohydrate (18). Five grams of α-benzoylarginine ethyl ester hydrochloride were dissolved in absolute methanol previously saturated with dry NH_3 at 0° C., stoppered, and let stand in the cold room at 4° C. for 3 days. The solution was concentrated *in vacuo* at 40° C. The amide solidified upon concentration. It was dissolved in 95% ethanol and precipitated with ether. The amide hydrochloride was washed several times with ether and recrystallized from hot 95% ethanol.

$C_{13}H_{22}ClN_5O_3$. Calculated. Cl 10.7, N 21.1
Found. Cl 10.7, N 21.1

L-Lysine Methyl Ester Dihydrochloride (20).

Seventeen grams of L-lysine monohydrochloride were dissolved in 340 ml. of absolute methanol previously saturated with dry HCl gas. The solution was again gassed with dry HCl without cooling. The solution was stoppered and let stand at room temperature for 24 hours. Crystallization of the ester salt began upon cooling and was completed by slowly adding ether (300 ml.). The ester salt was washed with ether and recrystallized from methanol-ether. The ester dihydrochloride can also be recrystallized from aqueous ethanol-acetone. M. p., 218° C. with decomposition (lit. 218° C. with decomposition).

$C_7H_{18}Cl_2N_2O_2$. Calculated, Cl 30.4; found Cl 30.5

DL-Phenylalanine Ethyl Ester Hydrochloride (21).

Five grams of DL-phenylalanine were suspended in 50 ml. of absolute ethanol and gassed with dry HCl for 10 minutes without cooling. The solution was then refluxed for 2 hours, gassed again and refluxed another hour. The solution was placed in a vacuum desiccator over P_2O_5 and a vacuum maintained until the entire reaction mixture solidified. The product was washed with ether and recrystallized from ethanol-ether. Yield, 4 g. M. p., 127° C. (lit. 127° C.).

$C_{11}H_{16}ClNO_2$. Calculated, Cl 15.4; found Cl 15.4

Benzoyl-DL-Phenylalanine (22). This is a general method of benzoylation of all amino acids which can yield only a monobenzoyl derivative and which are stable under the conditions of the reaction. The method is based on the findings of Carter and Stevens, namely, that benzoylation occurs most efficiently with excess benzoyl chloride, and under conditions of rapid hydrolysis, that is, dilute solution and high basicity. 16.5 g. (0.1 mole) of DL-phenylalanine were dissolved in 75 ml. of 2 N NaOH and 25 ml. of water were added. In alternate portions 23 ml. (0.2 mole) of benzoyl chloride and 230 ml. of 2 N NaOH were added with vigorous stirring over a period of 1 hour. The benzoyl chloride was added in about 5 ml. portions. The reaction mixture was kept very basic at all times and the temperature was not allowed to rise over 30°C. After addition of the reagents the reaction mixture was stirred for 1 hour longer. The solution was then acidified to Congo Red dropwise with 34 ml. of concentrated HCl under stirring and cooling whereupon the reaction mixture solidified to a white mass. The product (benzoylphenylalanine and benzoic acid) was filtered off, washed with cold water and dried in air. Then the product was washed well with 600 ml. of ether in portions to remove the benzoic acid. The crude product was used in subsequent preparations. Yield, 20 g. M. p. 184-186° C. (lit. 185-186° C.).

Benzoyl-DL-Phenylalanine Ethyl Ester (27). Seven grams of benzoyl-DL-phenylalanine were dissolved in 55 ml. of absolute ethanol and gassed to saturation with dry HCl without cooling. The solution was then refluxed for 2 hours, gassed again and refluxed another hour. After cooling, the flask was placed in a vacuum desiccator over P_2O_5 and suction applied. Upon concentration in this manner the ester crystallized out. It was filtered off, washed well with cold water, dried and recrystallized from aqueous ethanol. The ester crystallized as long white needles. M. p., 94-96° C. (lit. 94° C.).

$C_{18}H_{19}NO_3$. Calculated, N 4.7; found N 4.7

Benzoyl-DL-Phenylalaninamide (26). Two grams of benzoyl-DL-phenylalanine ethyl ester were dissolved in 50 ml. of absolute methanol previously saturated with dry NH_3 at 0° C. The flask was stoppered and allowed to stand at room temperature for 2 days during which time the amide crystallized out in white fluffy needles. These were filtered off, dried in air and recrystallized from hot $CHCl_3$. The amide crystallized in white needles. M. p., 195-197° C. (lit. 197-198° C.).

$C_{16}H_{16}N_2O_2$. Calculated, N 10.4; found N 10.4

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