THE PROTEOLYTIC ACTIVITY OF A THERMOPHILIC BACTERIUM

by

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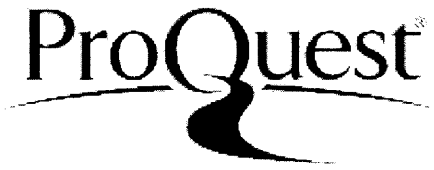

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INTRODUCTION

The manner in which bacteria degrade proteins has been investigated by bacteriologists for a period extending over 60 years. In this field of endeavor, numerous organisms have been examined for their ability to utilize proteins. The studies have been concerned with the ability of bacteria to decompose proteins directly as a source of nitrogen, carbon, and energy requirements. The problem has been examined in a more indirect manner by growing the bacterium in a medium, obtaining a cell-free filtrate, and testing this cell-free filtrate for proteolytic activity on an external protein substrate.

The thermophilic bacteria have been only superficially examined in this respect save for the isolated studies of Levine and Shaw (1924) and Chopra (1945, 1946a, 1946b). More specifically the factors which govern the proteolytic activities of a thermophilic organism when grown directly on a native protein have never been resolved. For this reason the present investigation was undertaken in an effort to establish the factors which influence the direct decomposition of a native protein by an obligate thermophilic bacterium.

II

HISTORICAL

The literature on the subject of bacterial proteolysis reveals that two main routes of investigation have been pursued by the workers in this field:

(1) The bacterial culture filtrates were tested for proteolytic activity by their action on proteins-indirect method.

(2) The bacteria were grown directly on the test proteins and their proteolytic activity tested thereon-direct method.

The preponderant amount of available data is concerned with the indirect method of determining the activity of filterable proteases, while few investigators have examined the direct effect of living bacterial cells on protein substrates.

Among the first workers in the field were Brunton and Macfadyen (1889) who noted that bacterial filtrates were capable of liquefying fibrin and gelatin. Fermi (1890) demonstrated conclusively that an active proteolytic enzyme could be obtained from a sterile cell-free filtrate of the medium in which a bacterium had grown.

Bainbridge (1911) reported on the direct utilization of pure proteins by aerobic bacteria. The proteins consisted of egg albumin, alkali egg albumin or horse serum in 0.1-0.6 per cent concentrations, which were combined with a simple inorganic salt base. Some of the bacteria tested were B. coli communis, B. proteus, Staphylococcus aureus, and B. typhosus.[1] Utilization of the protein by the organisms was indicated.

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[1] Bacterial nomenclature in this thesis is written in the same manner as in the original reference. Transposition of the nomenclature into the modern terminology is rendered too uncertain due to the absence of any universally accepted system of nomenclature in the early years of bacteriological literature.

In this reference and others the generic names of some bacteria are abbreviated and are not recorded in their entirety in any portion of the references.

by the increase of the bacterial population as determined by plate counts. Proteolytic activity data was obtained by precipitation of unaltered protein with tannic acid and weighing, and also by Kjeldahl determinations of solubilised protein. He observed that when the inocula were small there was some evidence of bacterial multiplication. When the inocula were large however, the bacterial numbers diminished. This was assumed by him to be due to the presence of trace amounts of non-protein nitrogenous matter in the medium. Glucose concentrations from 0.5-1.0 per cent had little influence on protein utilisation. He concluded that the organisms could not utilize the native protein as a nutrient when it represented the sole nitrogen source.

Drummond (1914) became interested in an organism resembling Proteus sp. which he isolated from sewage sludge. It liquefied gelatin rapidly and was capable of secreting the enzyme gelatinase when grown in nutrient gelatin, peptone water or peptone broth.

In 1915 Sperry and Hettger conducted a study on the growth of bacteria on purified proteins, which was an enlargement on the previous work by Rainbridge. The purified proteins consisted of egg albumin, serum albumin, and edestin which were sterilised by filtration through a Berkefeld filter. Concentrations of 0.2-0.8 per cent of the proteins were mixed with a suitable salt solution and served as the medium. Proteolytic activity was chiefly measured in terms of increase in the bacterial population. The activity was further tested by precipitating the residual protein and weighing. Thirteen organisms were tested including B. anthracis, B. prodigiosus, B. pullorum, and B. proteus vulgaris. They concluded that the bacteria were unable to decompose

proteins, whether animal or vegetable in origin, and that proteolysis could occur in the presence of peptone or some other nitrogenous material.

Diehl (1919) reported on the activity of culture filtrates of B. pyocyaneus, B. prodigiosus, and B. subtilis, on casein and gelatin. When grown on media lacking organic nitrogen, the organisms produced no protease in the culture filtrates. However, when grown on gelatin, casein, and nutrient broth, the culture filtrates contained enzymes which would digest both gelatin and casein. The test method consisted of adding aliquots of the cell-free filtrates to the test protein and when suitable periods had elapsed, the residual protein was precipitated by acid and then weighed.

Levine and Carpenter (1923) studied the activity of 7 bacterial species when grown on a medium consisting of 3.5 per cent gelatin supplemented with 0.1 per cent peptone. Proteolytic activity was determined by formol titrations and viscosity measurements. Three types of action were identified:

(1) The gelatin was not liquefied.
(2) The gelatin was partially liquefied and accompanied by slight increases in the formol titration.
(3) The gelatin was completely liquefied and accompanied by marked increase in the formol titration.

Generally the viscosity was found to drop before the formol titration began to rise, and the ability to liquefy gelatin was not always associated with ability to decompose the gelatin.

Waksman and Lomanitz (1925) made a detailed study on the proteolytic activity of B. cereus and B. fluorescens when cultured on casein. The medium consisted of a 1.0 per cent casein combined with an inorganic salt solution. The casein was employed as the sole source of nitrogen, or as the combined nitrogen and carbon source. Analytical data were obtained for total nitrogen by the Kjeldahl method, ammonia nitrogen by

the Folin method, and amino nitrogen by the van Slyke technic. The data obtained from these experiments showed that B. cereus could decompose casein most rapidly but that B. fluorescens was unable to decompose casein.

In 1926 Kendall and Keith studied the proteolytic action of B. proteus filtrates on a gelatin substrate. They compared this activity with that of pepsin and trypsin and found that the bacterial enzyme was most active. Further when the organism was grown in a medium containing utilizable carbohydrate, the enzyme although present in the filtrate, was found to be in an inactive state.

Merrill and Clark (1928) reported on the factors which affect gelatinase production by Proteus sp. when grown under various conditions of incubation. The influence of acidity, aeration, the presence of calcium and magnesium salts, and the composition of the medium was studied in detail. Proteolysis was tested by growing Proteus sp. on the test medium, removing an aliquot and mixing with gelatin for a given period, then noting the jelling time of the gelatin. It was observed that a peptone medium at pH 7-8 resulted in optimal gelatinase production. The production of gelatinase was found to increase with increased aeration, and active proteolytic filtrates could be obtained from synthetic media in the presence of calcium and magnesium salts. They also noted in many instances that even though a medium was capable of supporting good growth of the organism, yet no gelatinase activity could be detected.

In 1930 Wilson studied the influence of medium composition on protease production by Bacillus subtilis and Pseudomonas aeruginosa. The activity of the cell-free filtrates was tested on gelatin and casein. She noted that protease production by these bacteria was a process of the

cell metabolism which was independent of the ingredients of the medium except insofar as these inhibited or stimulated growth. Thus any medium, whether synthetic or non-synthetic, induced good activity provided that it supported an adequate bacterial growth.

The influence of calcium and magnesium salts on protease production has been reported in a series of papers by Haines (1931, 1932, 1933). Ps. fluorescens, B. proteus, and Achromobacter sp. were found to produce proteases in a synthetic medium provided both calcium and magnesium salts were present. Results compared equally well with protease production in broth cultures.

The ability of bacteria to utilize purified plant proteins such as zein, gliadin, and edestin as a sole source of energy and nitrogen was investigated by Waksman and Starkey (1932). Organisms such as B. mycoides, B. subtilis, Sarcina lutea, and Ps. fluorescens were grown on a medium consisting of an inorganic salt base in combination with 0.5-1.0 per cent of the test protein. All organisms were found to decompose the purified plant proteins readily.

Console and Hahn (1938) observed that the rate of gelatin decomposition by B. subtilis continued to increase for at least 5 days after maximal populations had been achieved. Five ml aliquots of whole broth cultures were added to 350 ml of a 5.0 per cent gelatin solution and activities were measured by the formol titration. They observed that the activity of a cell-free filtrate was found to compare equally well with the activity of the whole culture from which it was obtained, and assumed that the proteolysis was partly due to an extracellular enzyme. They concluded that no close relationship existed between the numbers of cells and the amount of gelatin decomposed.

Bacillus subtilis was also investigated by Stockton and Wyss (1946). This organism was found to have a requirement for manganous sulfate when grown on nutrient broth in order to elaborate protease.

Mead and King (1929) investigated the ability of Clostridium sporogenes and Cl. histolyticum to degrade fibrin and edestin in the presence of meat extract. The proteolytic activities of the organisms were observed when cultured on a medium which consisted of 4.0 g of protein and 0.12 g of meat extract in a total volume of 35 ml. The analytical procedure consisted of analyses of tyrosine, histidine, and ammonia nitrogen as well as the formol titration for amino nitrogen. These workers noted that Cl. histolyticum exhibited a greater proteolytic action than Cl. sporogenes. This greater activity varied with the protein used. They also found that both organisms were capable of utilizing tyrosine to a greater degree than histidine.

Kochalaty and Weil (1932) found that altering the pH of the medium resulted in a change in the pH optimum of the protease secreted by Cl histolyticum. In a later paper, Weil and Kochalaty (1937) indicated that the protease of Cl. histolyticum showed optimal activity at pH 7.0. In addition, this activity was found to be stimulated in the presence of sulfhydryl compounds in conjunction with the divalent ions of iron, manganese, nickel, and copper at low concentrations. Further studies by Kochalaty, Weil, and Smith (1936) proved that the activation behavior of the ferrous ion and cysteine combination was operative under all the conditions examined. In another report by Weil, Kochalaty, and Smith (1939) the scope of the investigation was enlarged to include Cl. sporogenes, Cl. welchii, Cl. putrificum, and Cl. botulinum. Once again it was found that the combination of the ferrous ion and cysteine gave

maximum activation when added to the protease filtrate. These results were partially confirmed by van Heyningen (1940) who observed that the extracellular protease of Cl. histolyticum was activated by cysteine.

Levine and Shaw (1924) studied the proteolytic activity of 4 unidentified thermophilic organisms. The organisms were cultured on 10 per cent gelatin supplemented with 0.1 per cent peptone. The decomposition of the gelatin medium by the thermophils was determined by formol titrations and viscosity measurements. Marked proteolytic activity was noted for all the thermophilic organisms.

Chopra (1946) examined the influence of carbohydrate on the production of protease by a group of thermophilic organisms including B. thermophilus, B. aerothermophilus, and B. thermoacidurans. When these organisms were grown on a 2 per cent carbohydrate broth and the cell-free filtrate tested for proteolytic activity on gelatin, a marked decrease in protease activity was noted. The carbohydrate broth culture was then centrifugated and the bacterial cells separated from the filtrate. He then tested the proteolytic activity of the whole culture, the cells and the cell-free filtrate. Marked activity was found to reside in the whole culture and in the cells but not in the cell-free filtrate. He concluded that although the bacteria had elaborated protease in the carbohydrate medium, the enzyme was intimately combined with the cellular constituents and was not released to the filtrate when the latter was tested for activity.

In another paper Chopra (1946a) compared the proteolytic activities of filtrates from B. thermophilus, B. aerothermophilus, and B. thermoacidurans. The culture filtrate and protein substrate were combined and incubated at 40 C and proteolytic activity was determined by means of viscosity measurements and van Slyke analyses for amino

nitrogen. He found that the thermophils produced active proteases which could be detected in the culture filtrates. Optimal activity was noted when growth occurred in the range pH 7-8.

The origin of protease derived from thermophils was investigated by Chopra (1946b). He found that the enzyme was endocellular in character and appeared in the culture filtrate only as a result of the autolysis of the cells. Calcium and magnesium salts were observed to activate the protease rather than to stimulate the quantity of the enzyme produced by the thermophils.

A recapitulation of the literature reviewed indicates that the problem of bacterial proteolysis has been examined in two general ways. Either the entire culture or the cell-free filtrate has been examined for proteolytic activity. The fact that both approaches have been employed by the workers in the field makes it somewhat difficult to evaluate the results.

Aerobic, anaerobic, and thermophilic bacteria have been examined for proteolytic activity. Workers have used such proteins as gelatin, casein, fibrin, egg albumin, horse serum, serum albumin, edestin, zein, and gliadin in experiments. Some analytical procedures which have been employed include the Kjeldahl determination for total nitrogen, van Slyke analyses and formol titrations for amino nitrogen, residual protein and ammonia nitrogen determinations, and viscosity measurements.

The evidence from the literature indicates that some bacteria will degrade native proteins when these are used as the sole source of nitrogen. Other organisms have been reported which will not degrade native proteins under the same conditions. These negative results are now subject to reevaluation in view of the newer knowledge of the vitamin

requirements of bacteria. The hydrolytic power of an organism varies with the protein tested. An organism may be capable of degrading one protein but not another.

In general most of the organisms investigated in the literature were capable of producing proteases in simple culture media. The enzyme formation was favored by a neutral or slightly alkaline reaction of the medium. Unfortunately much of the early work was done when the concept of pH and its application to bacteriology had not yet been formulated, consequently some of the early results may be justifiably questioned. Protease production could occur in a synthetic medium in the presence of calcium and magnesium salts. These salts in a non-synthetic medium appeared to activate protease activity. The ferrous ion in combination with cysteine appeared to stimulate the proteolytic activity of the anaerobic bacteria. The presence of dextrose in a medium had either little or an adverse effect on proteolytic activity. In the case of the thermophils at least, it was shown that the presence of carbohydrate resulted in the formation of sufficient protease, but that this protease was securely bound by cellular material and not released to the medium.

In the early researches, the increase in the bacterial population when grown on a native protein was used as an index of protein utilization, with the implication that proteolysis had occurred. The evidence from the literature indicates that no strict correlation exists between bacterial numbers and proteolytic activity. In general the data suggest that the formation of protease is usually accompanied by the development of fairly large bacterial populations.

III

MATERIALS AND METHODS

Cultures. The organism was a "flat sour" type of thermophil originally isolated from peas and obtained from the National Canners Association, bearing the designation N. C. A. 1503. It was a gram-positive, sporogenous rod growing at 55-65 C, but not at 37 C. The organism was an obligate thermophil or stenothermophil (Imsenecki and Solnzeva 1946). It was chosen from a group of 11 obligate thermophils on the basis of its ability to decompose protein substrates as determined by visual examination. The protein substrates included gelatin, Holman's alkaline chopped meat, litmus milk, egg albumin, liver, and beef blood serum.

Glassware. The glassware including Roux culture bottles, pipettes, 4 and 32 oz screw-cap bottles, volumetric flasks, porcelain filter funnels (Selas No. 02), sintered glass filters (Corning [illegible] F) were all chemically cleaned with aqua regia. Mandler filters (Nos. 8, 15) were washed in acidified 0.1 per cent potassium permanganate solution, followed by a 0.2 per cent oxalic acid solution. All glassware was finally rinsed with distilled water and sterilized when required, either in the autoclave or in the hot air oven in the customary manner.

Incubation. A double-walled air incubator was used for all culture work at 55 C $\pm$ 0.1 C. National Bureau of Standards tested thermometers were interspersed with the culture flasks to check the temperature.

Preparation of Inoculum. The stock cultures and inocula were grown on media of the following composition: tryptiease, 2.0 per cent; yeast extract, 0.3 per cent; NaCl, 0.6 per cent; K_2HPO_4, 0.1 per cent; agar, 1.6 per cent. Casitone was occassionally substituted for tryptiease and

employed in the same concentrations. Cultures were grown on the stock medium agar slants and transferred daily for 3 days prior to preparation of the inoculum. The growth from 6-8 slants was harvested and suspended in sterile distilled water. The bacterial cells were then pooled and centrifugated at 2500 r p m for 20 minutes. This washing process was repeated 3 additional times and the final centrifugate taken up in 15-20 ml of sterile distilled water. This suspension constituted the inoculum which was delivered in 1 ml amounts into Roux culture flasks or 4 oz screw-cap bottles containing the media.

Preparation of Test Media Constituents.

Stock Casein Solution. Vitamin-free casein (Control Nos. 3860, 9601) was obtained from the Nutritional Biochemicals Corporation. It was prepared in 5 and 10 per cent concentrations in the following manner. Fifty or 100 g of casein was added to a 2 L beaker containing 1000 ml of M/15 Sorensen's phosphate buffer pH 7.0 (see below). The addition of the casein was accomplished in a stepwise manner to avoid clumping of the casein particles, and in addition an air-powered stirrer aided in the solubilizing process. When the particles were thoroughly "wetted", the suspension was transferred to a 2 L Erlenmeyer flask and the flask was stoppered. The solution was next gently heated for a short while in the Arnold sterilizer until completely dissolved. The casein solution was then adjusted to pH 7.0 by the addition of 40 per cent NaOH and filtered through filter paper. Once again the casein solution was returned to the Arnold sterilizer together with a completely assembled Mandler filtration apparatus and both heated for a short period of time until hot. The filter apparatus was then removed, swathed in towels to prevent rapid loss of heat, and attached to the vacuum line. The casein solution was then removed from the Arnold and similarly swathed to prevent heat loss. The

casein solution was then filtered as rapidly as possible. It was found from experience that heating prior to filtration prevented clogging of the filter and enabled complete filtration of the casein. This was undoubtedly due to the fact that the viscosity of the hot casein was considerably lower than the cold casein solution thereby permitting rapid passage through the filter. Sterility checks were accomplished by culturing 10 ml aliquots of the casein solution in thioglycollate medium at 55° C.

Salt Mixture. The following salts were dissolved in 500 ml of distilled water without heat: H_3BO_3, 25 mg; KI, 2.5 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; $FeSO_4 \cdot 7H_2O$, 500 mg; $MnSO_4 \cdot 4H_2O$, 500 mg; $CuSO_4 \cdot 5H_2O$, 2.5 mg; $ZnSO_4$, 2.5 mg. This salt mixture was filtered through sintered glass filters (Corning UF) and when used in the experiments was dispensed in 0.5 ml amounts per 100 ml of medium (Cleverdon 1949).

Supplemental Solutions. Stock solutions of dextrose, yeast extract, amino acids, vitamins, peptides, calcium chloride, magnesium sulfate, purines, and pyrimidines were prepared in distilled water in concentrations such that when 1 ml of the stock solution was added to 100 ml of the medium, the desired concentration of the substance was obtained. These solutions were all sterilized by filtration through sintered glass filters.

Buffer Mixtures. The major portion of the media was prepared using the Sorensen phosphate buffer system (Clark 1928) which consisted of the following: (1) A M/15 solution of primary potassium phosphate which contained 9.078 g of KH_2PO_4 in 1 L of solution. (2) A M/15 solution of secondary sodium phosphate which contained 9.44 g of anhydrous Na_2HPO_4 in 1 L of solution. For a solution at pH 7.0, four volumes of (1) were combined with 6 volumes of (2). In a medium where a considerable volume

of supplemental substances was added, the resulting dilution was compensated for by the addition of more concentrated phosphate salts such that the end concentration in the medium was M/15 strength at pH 7.0. The following buffer systems were also employed in the course of the work: Sorensen's citrate buffer (Clark 1928) consisting of 0.1 M disodium hydrogen citrate and 0.1 M Na_2OH at pH 7.0; Clark and Lubs buffer system consisting of 0.1M boric acid (0.1M KCl) and 0.1N Na_2OH at pH 8.0 (Lange 1939); and the Prideaux-Ward buffer system made up to pH 7.0 and consisting of 0.04 M H_3PO_4, 0.04 M phenyl acetic acid, and 0.04 M boric acid, partially neutralized with 0.2 N Na_2OH. All buffer solutions were sterilized by filtration through a Selas No. 02 porcelain filter.

Plate Counts. Bacterial populations in various media were determined by pouring duplicate plates of each dilution, using the yeast extract, trypticase agar medium. In practice the plates were prepared in the customary manner and immediately upon hardening were placed in metal petri plate containers to retain moisture. The containers were then placed in the 58 C incubator for 48 hours ± 3. Following incubation, colonies were counted by means of a Quebec colony counter and a Veeder Root hand tally.

Microscopic Counts. Bacterial smears were made in the customary manner; 0.01 ml of the sample being spread over 1 square cm. The smear was stained with methylene blue and 12 fields were counted per sample by means of a microscope using the oil immersion lens.

Pancreatin Solution. Solutions of pancreatin (Pancreatin Merck U.S.P. Control No. 43546) were prepared in 100 mg, 200 mg, and 800 mg amounts in 10 ml of Sorensen phosphate buffer at pH 8.0. Control pancreatin solutions were prepared in the same manner and inactivated

by steaming in the Arnold steriliser for 25 minutes. The casein and enzyme solutions were combined and overlayered with toluene and incubated at 40° C for stated lengths of time. The pH of the enzyme mixture was checked daily by means of indicator paper and maintained at pH 8.0-9.0.

Casein Hydrolysate. The acid hydrolysis of 100 ml of a 2.5 per cent casein solution in Sorensen phosphate buffer at pH 8.0 was accomplished by the addition of 12.5 ml of 20 per cent HCl and refluxing the mixture for 24 hours. The HCl was then removed by distillation under reduced pressure and the final volume made up to 100 ml.

Formol Titration. The formol titration of Sorensen (1907) was used in modified form throughout the course of the experimentation to determine proteolytic activity of the bacterial cultures grown directly on the casein substrate. This titration procedure is capable of determining such substances as ammonia, primary amines, the amino groups of amino acids and polypeptides. Brown (1923) modified the procedure to minimize the errors which would be caused by the presence of such interfering substances as carbonates and phosphates. In 1933 van Slyke and Birk studied the influence of preliminary neutralization on amino acid mixtures at pH 7.0 with an end point in the titration at pH 9.0. Levy (1954) subjected the formol titration to an analysis and from his experimental data concluded that maximum accuracy was obtained when: (1) The formaldehyde concentration is 6-9 per cent at the end volume, (2) Neutral (pH 7.0) formalin is used, (3) The concentration of the amino acids is as high as possible, (4) An average titration end point for the amino acid mixture is used.

The high degree of precision obtainable with the formol titration is described by Dunn and Loshakoff (1936). These authors employed a glass electrode in their titration of pure amino acids and dipeptides and achieved an accuracy of ± 0.1 per cent.

Method of the Formol Titration. The formol titration in its modified form was employed throughout the course of the work and performed in the following manner: (1) A Beckman pH meter, model H_2 (National Technical Laboratories) with extendable electrodes (especially the sleeve type calomel electrode) in combination with a Varitan transformer, model V-1 (United Transformer Company) set for 110 volts, was used throughout the course of the experiments, (2) A 10 ml burette calibrated in increments of 0.05 ml was employed to deliver the 0.05 N NaOH solution which was used in the titrations, (3) Merck neutral formaldehyde, assaying at 36-38 per cent, was poured into a large beaker and adjusted to pH 7.0 with alkali by means of the Beckman pH meter, using an electric stirrer. This neutralization procedure when augmented by vigorous stirring for 15-20 minutes resulted in a very stable formaldehyde which maintained its neutrality for several hours, (4) Five ml of the neutral formaldehyde was then pipetted into a 50 ml beaker, to which was also added 15 ml of distilled water and the contents thoroughly mixed. The mixture was then titrated with 0.05 N NaOH to pH 9.5. This process was repeated several times and the results were averaged. The mean obtained was the formaldehyde blank value which was subtracted from all subsequent titrations, (5) One ml of the unknown sample was pipetted into a 50 ml beaker together with 14 ml of distilled water and thoroughly mixed. Then with either dilute acid or alkali, the mixture was adjusted to pH 7.0, the beginning point of the titration, (6) To this solution at pH 7.0, 5 ml of the adjusted formaldehyde was added and thoroughly mixed until the pH meter indicator came to final rest, (7) This mixture was titrated with 0.05 N NaOH to end point pH 9.6 (Welnick and Oser 1949), (8) The formaldehyde blank value of (4) was subtracted from the titration value obtained in (7). This gave the formol titration

of 1 ml of the sample in terms of ml of 0.05 N N_aOH, (9) In terms of titrable nitrogen, 1 ml of 0.05 N N_aOH is equivalent to 0.7 mg of nitrogen.

IV

RESULTS AND DISCUSSION

The factors influencing the proteolytic activity of the experimental thermophilic organism (N.C.A. 1503) which were examined in the course of this work may be conveniently divided in the following manner:

I Physical Factors
- A. Temperature of Incubation 45 C
- B. Length of Incubation
- C. pH
- D. Oxygen Tension
- E. Types of Buffers
- F. Concentration of Casein
- G. Different Lots of Casein

II Chemical Factors
- A. Basal Medium Constituents
  1. Dextrose
  2. Salt Mixture
  3. Thiamin, Niacin, Biotin
- B. Calcium and Magnesium Salts
- C. Vitamins
- D. Amino acids, Purines and Pyrimidines
- E. Dipeptides
- F. Peptone and Amino acid Mixtures
- G. Comparison of Acid, Enzymatic, and Bacterial Digestions

III Biological Factors
- A. Nature of the Organism
- B. Different Types and Concentrations of Inocula
- C. Relation of Bacterial Numbers to Proteolytic Activity

The data obtained in all the experiments were derived from the formol titration which was described in the section on Materials and Methods. At all times the manner of performing the titration was rigorously adhered to, and all the recorded results are in terms of ml of 0.05 N $NaOH$.

To determine the proteolytic activity of the thermophil in a particular medium, two flasks containing the identical medium, one of

which was inoculated (the culture) and the other uninoculated (the control) were incubated at 55 C. At stated intervals 1 ml aliquots from both the culture and the control were subjected to the formol titration. If the formol value of the control were subtracted from the formol value of the culture, the difference represented the proteolytic activity of the thermophil on the particular medium. Further if this difference were divided by the days of incubation required to achieve this difference, one would now have an average daily rate of change (activity units) of the proteolytic activity for the time interval in question. Almost all of the data of the proteolytic activity of the organism has been recorded as the difference between the culture and its control at the end of a time interval (formol titration difference).

I Physical Factors

A. Temperature of Incubation 45 C. The organism was known to grow at 55 C and 65 C but not at 37 C; it was decided to test the influence of an intermediate temperature such as 45 C on the proteolytic activity of the bacterium. This was done for two reasons: (1) Chopra (1946a) although he cultured thermophilic organisms in broth at 50 C, nevertheless tested the proteolytic activity of the cell-free filtrates on protein substrates at 40 C with good results; (2) Tauber (1949) observed that above 50 C enzymes in solution were rapidly inactivated, which destruction increased with elevation of the temperature and was complete at 80 C. Presumably if the test organism could be cultured at 45 C, less inactivation of the enzymes would occur than at 55 C. Two similar sets of 0.5 per cent vitamin-free casein were prepared which varied as to constitution. Both sets were inoculated with a suspension of the organism containing 320,000 organisms per ml of the medium. One

set was incubated at 45 C and the duplicate set at 55 C. Formol titrations were made at intervals and recorded. The difference in activity between the two sets was visually evident. The 55 C set showed the typical pattern of bacterial activity in the medium which was observed throughout the entire course of all the experiments, and for this reason is described in detail. The light amber colored medium at the end of 2-3 days showed the typical cloudiness and "swirling" of bacterial growth when shaken, as compared with the clear amber controls. After 4-6 days of incubation, the culture medium turned a complete milky white with evidence of sedimentation. By 8-10 days of incubation the milky white color of the medium was completely dissipated leaving only some sediment at the bottom of the culture flask. Thereafter the sediment and the culture fluid burned a dark brown color with extended incubation. It was usually after the disappearance of the milky white color that proteolytic activity could be detected by the formol titration.

The 45 C set of cultures showed none of this activity; indeed there was no evidence of growth of the inoculum whereas in the 55 C set, the count in one culture was as high as 18,000,000 per ml in 25 days. Similarly formol titrations indicated no evidence of proteolytic activity in the 45 C set but in the 55 C set, one culture was as high as 0.64 ml formol titration difference in 38 days.

8. Length of Incubation. The period of incubation of any series of test media was determined by indications of proteolytic activity. Generally when significant formol titration differences were obtained among the cultures in any given series, the proteolytic measurements for the series were then terminated. The results were evaluated within the series with respect to the stimulation of proteolytic activity which any particular medium possessed.

On the other hand it was found that significant formol titration differences among different experimental series occurred at the end of different time intervals. This was due to several factors such as different conditions of aeration of the cultures, different concentrations of casein, different media constitution, and the variable survival capacities of fresh inocula of the organism.

The effect of aeration, casein concentrations, and medium constitution on the length of the incubation period will be discussed below. The question of survival capacities of the freshly inoculated organism presented a serious problem. It was considered of prime importance to use minimal numbers of the organism as inoculum to initiate growth within a reasonable period, since it was realized that the addition of too massive an inoculum would possibly result in the fortuitous introduction of the very factors, the influence of which it was desired to study. In addition the ability of thermophilic bacterial populations (inocula) to survive for any extended period at a temperature of 55 C, whether cultured upon favorable or unfavorable media, is not of a very high order. Hansen (1953) observed that the death rate for a facultative thermophil was maximal in the 60 C range. Indeed two population plots at 62.5 C and 57.5 C were shown by him to have already completed the logarithmic death phase of the growth curve within 25 hours. Similarly Allen (1950) noted a drop in numbers of viable facultative thermophils from 10^8 to 10^3 per ml in 2.5 hours when maintained at 65 C. Chopra (1946a) in his study on thermophilic organisms states:

> "Death of the cells as determined by failure to multiply starts quite early after inoculation, in fact multiplication and death may be said to begin almost simultaneously; it is therefore inaccurate to say that in young cultures autolysis does not take place."

It is seen therefore that in any particular experimental series comprising a number of culture flasks each containing a different culture medium, the effect on the survival on the organism will be variable depending on the medium make-up and the innate "suicidal" tendencies of the organism itself. If the death rate of the inoculum population after seeding were excessive, then other factors being equal, a belated growth response with subsequent belated proteolytic activity was to be expected. Consequently if a sufficient incubation period were permitted in which the complete growth pattern were allowed to run its full course, then sufficient formol titration differences could be obtained upon which to form an opinion regarding the efficacy of the media involved.

Thus it was found throughout the course of the work that two identical experimental series when run at different times could not be compared in a strict manner, since maximal activities did not always occur in the same degree at the same time. However if one medium composition were found to excell all others in its series at a certain activity level at a certain time, then this same medium composition would excell all the others in a repeat series at a later date, although the activity and time values might vary somewhat from the first trial. In all the following experiments, incubation periods were permitted to continue from 2-8 weeks before terminating the experiments.

C. pH. The starting pH of the medium which was to be inoculated with the test organism exerted a definite influence on the proteolytic activity of the thermophilic organism. Hansen (1933) noted that a pH of 4.7 had a deleterious effect on the populations of a facultative thermophil which was not evident in a medium maintained at neutrality with $CaCO_3$. Chopra (1946a) studied the influence of pH on the proteolytic

activity on gelatin of thermophilic cell-free filtrates. He employed the McIlvaine citrate phosphate buffer system with an incubation at 40 C. It was found that the optimal range lay from 7.6 to 8.3.

To digress for a moment it may be noted in contrast that the test organism was incapable of growing at 40 C and also unable to initiate growth in a citrate phosphate buffer medium. It would seem that the citrate phosphate buffer may prevent the growth of a thermophilic organism such as N.C.A. 1503 and thereby prevent protease formation. But once protease is elaborated by a thermophilic organism, the proteolytic activity thereof may be unhindered in the presence of citrate phosphate buffer.

It is of interest to consider the basic differences underlying the fundamental approaches to the entire problem of proteolysis namely the direct and indirect methods of analysis. Obviously the factors which favor the proper functioning of a protease system once it has been elaborated by an organism and tested by the indirect method, may actually prove unsuitable or inimical to the development of the living organism itself.

The influence of pH on the proteolytic activity was examined. The medium on which the thermophilic organism was cultured was as follows: vitamin-free casein, 0.6 g; salt mixture, 0.6 ml; yeast extract (Difco), 0.01 g; dextrose, 0.1 g; thiamin, 100µg; niacin, 100µg; biotin, 4.0µg; Sorensen phosphate buffer pH 6.85-7.3; and Clark-Lubs buffer pH 7.6-8.65. The total volume was 100 ml and the pH of the media were respectively as follows: 6.65, 6.0, 6.4, 6.82, 7.0, 7.3, 7.6, 8.35, 8.65. The media were dispensed in 100 ml amounts in rubber-stoppered Roux bottles laid on end and incubated at 65 C. The results are presented in table 1, where it is seen that optimal points occur between pH 6.4-6.8 and pH 7.3-7.6.

TABLE 1

Influence of pH on the proteolytic activity of t
of the test organism

	Culture Designation								
	A	B	C	D	E	F	G	H	I
Initial pH	5.85	6.0	6.4	6.82	7.0	7.3	7.6	8.35	8.65
Formol Titration Difference after 35 days incubation	-.08	.10	.30	.27	.17	.24	.30	.04	.05

D. Oxygen Tension. This organism exhibited aerobic characteristics of growth, consequently it was considered desirable to determine the influence of oxygen tension on proteolytic activity. Furthermore an attempt was made to correlate oxygen tension with pH should any such relationship exist.

The oxygen tension of a medium will vary directly with the area of its surface exposed to the air, and inversely with its depth. Combined into one expression:

$$RO_2 = \frac{\text{surface area of medium}}{\text{depth of medium}}$$

Thus we have a simple expression describing the oxygen tension of the medium as the ratio of the surface area of the medium to the depth of the medium. Since the volume of the medium was 100 ml and was always constant in all the experiments, the RO_2 varied as a result of the size and shape of the culture flasks employed.

The medium for this experiment consisted of the following: vitamin-free casein, 0.6 g; salt mixture, 0.5 ml; thiamin, 100/µg; niacin, 100/µg; biotin, 4/µg; Biopar E (Armour), 0.01 g; Sorensen phosphate

buffer, pH 6.0-8.0; Clark-Lubs buffer, pH 9.0. The total volume was 100 ml. The media was made up in triplicate amounts at pH 6, 7, 8, 9. For each pH, the culture flasks were: 4 oz screw-cap bottle, RO_2=1.7; 1L Erlenmeyer flask, RO_2=74; 32 oz screw-cap bottle, RO_2=190. Thus a total of 12 culture flasks, all containing the same medium but varying as to pH and oxygen tension comprised the series. The results are noted in table 2 and figure 1.

It will be observed that all optima fell in the pH 6-7 range, (figure 1) regardless of the RO_2 value. RO_2 74 generally gave the best activity. Interestingly the results do show a close relationship between pH and oxygen tension. Further it is possible to supply too much oxygen to the detriment of the proteolytic action.

On the basis of these results it was decided to use the Sorensen phosphate buffer at pH 7.0 in future work. The possibility of using the 1L Erlenmeyer flasks, since these gave the best results had to be discarded. The reason for this was the large amount of incubator space which would have been required. A rubber-stoppered Roux culture bottle laid on one end having an RO_2 value of 42 was substituted instead and employed in all subsequent experiments.

E. Types of Buffers. In all the experimental, buffers were included for pH stability in the cultures (table 12) and for the greater ease of manipulation which they afforded in the formol titrations. Proteolytic activity could be observed with the buffer lacking. Although the Sorensen phosphate buffer was constantly employed in all the experiments, it was considered of worthwhile value to attempt the use of other buffers to see if proteolytic activity would be influenced. The Clark-Lubs buffer consisting of boric acid and sodium hydroxide, and the Prideaux-Ward buffer system consisting of phenyl acetic, phosphoric, and boric acids were substituted and proteolytic activity could be noted (table 2:K;

TABLE 2

Influence of pH and oxygen tension on the proteolytic activity of the test organism

	Culture Designation											
	A	B	C	D	E	F	G	H	I	J	K	L
RO_2*	1.7	74	190	1.7	74	190	1.7	74	190	1.7	74	190
pH	6.1	6.1	6.1	7.2	7.2	7.3	8.1	8.1	8.1	8.7	8.7	8.8
Formol Tit. Diff. after 53 days Incubation	.21	.77	.23	.30	.72	.50	.24	-.06	.14	.02	.35	.01

* RO_2=1.7 = 4 oz screw-cap bottle

RO_2=74 = 1 L Erlenmeyer flask

RO_2=190 = 32 oz screw-cap bottle

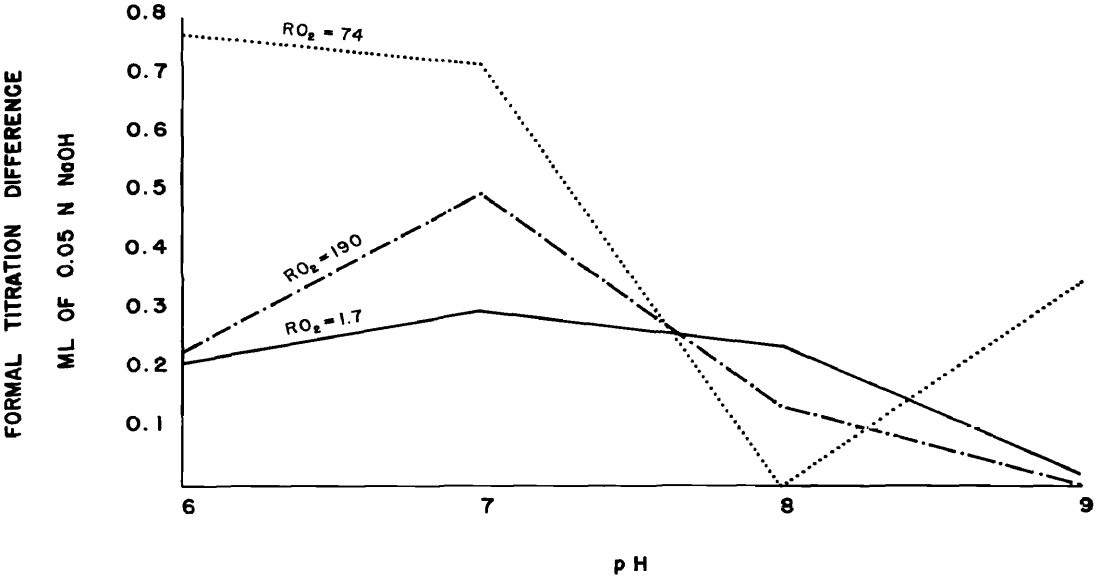

Figure 1. Influence of pH and oxygen tension on the proteolytic activity of the test organism after 65 days incubation.

F. Different Concentrations of Casein. Part of the experimental work entailed the use of a 0.5 per cent casein concentration. It was generally found that with this concentration, significant proteolytic activities did not develop until 4-6 weeks had elapsed. However, earlier responses (2-3 weeks) could be obtained when increased concentrations of casein were employed. A 2.5 per cent concentration of casein was found to be very satisfactory and was employed in subsequent experiments. Results on varying casein concentrations are noted in table 3.

G. Different Lots of Casein. When the supply of one lot of casein code 3860 was exhausted, another lot, code 9601 had to be used. When both codes were tested simultaneously for proteolytic activity under identical conditions, different formol values were obtained for similar concentrations. The results of this experiment are to be found in table 3.

TABLE 3

Influence of different lots (3860 and 9601) and concentrations of casein on the proteolytic activity of the test organism

	Culture Designation			
	A	B	C	D
Constituents/100 ml of medium				
Vitamin-free casein (Concentration and code as indicated)	2.5g (3860)	2.5g (9601)	1.0g (3860)	1.0g (9601)
Salt Mixture 0.5 ml	x	x	x	x
Dextrose 0.1g	x	x	x	x
Vitamins: Thiamin 100 µg, Niacin 100 µg, Biotin 4 µg	x	x	x	x
Sorensen buffer pH 7.0	x	x	x	x
RO_2 = 47	x	x	x	x
Formol titration difference after 16 days incubation	1.86	2.75	0.54	1.06

II Chemical Factors

A. Basal Medium Constituents.

1. Dextrose. Initially, a 0.5 per cent concentration of dextrose was included in the medium to serve the organism as a readily available source of carbon. The acidity resulting therefrom adversely affected the buffer system, and a 0.1 per cent concentration of dextrose was substituted instead. With respect to proteolytic activity, the presence of dextrose was found to be helpful (table 4: D, E).

2. Salt Mixture. The salt mixture, the composition of which has been described in the section, Materials and Methods, was found to have no effect on proteolysis (table 4: B).

3. Thiamin, Niacin, and Biotin. The test organism had been shown to exhibit good growth when cultured on casein hydrolysate supplemented with the vitamins thiamin, niacin, and biotin (Cleverdon, Pelczar, and Doetsch 1940). These three vitamins were employed throughout the course of the work. Examination of the data in table 4 indicates that thiamin results in better activity than either niacin or biotin (H, I, J). Further, when the activity of these vitamins are compared with the controls (A, C) it is observed that only thiamin (H) significantly affects proteolytic activity. The combined vitamins (F) are more effective than when used individually. When the complete basal combination of casein, salts, dextrose, and vitamins are combined, maximum activity is obtained (G).

B. Calcium and Magnesium Salts. Haines (1931, 1932, 1933) indicated that calcium and magnesium salts were of value in the production of proteases. It was endeavored to test these findings by the incorporation of $CaCl_2$ and $MgSO_4$ in M/250 concentrations in the casein

TABLE 4

Influence of various constituents of the medium on the proteolytic activity of the test organism

	Culture Designation									
	A	B	C	D	E	F	G	H	I	J
Constituents/100 ml medium										
Vitamin-free casein 2.5 g Sorensen phosphate buff. pH 7 NO_2= 47	x	x	x	x	x	x	x	x	x	x
Salt Mixture 0.5 ml		x					x			
Dextrose 0.1 g				x	x (0.5g)		x			
Thiamin 100 µg						x	x	x		
Niacin 100 µg						x	x		x	
Biotin 4 µg						x	x			x
Formol titration difference after 24 days incubation	0.12	0.15	0.16	0.30	0.47	0.47	0.88	0.36	0.18	0.02

medium. No significant improvement in the proteolytic activity could be detected at pH 7.0.

C. Vitamins. After the three vitamins thiamin, niacin, and biotin had been found to favor proteolytic activity, it was considered worthwhile to examine further the role of the entire vitamin B complex in this regard. The results of such an investigation are seen in table 5. It may be observed by referring to the formol titration differences that no combination of the vitamins showed any significant improvement over the activity of the three basal vitamins of Culture A. The calcium panthothenate (C) value is slightly greater than (A) but this is within the experimental error of the analytical technique. It can be seen that multiple quantities of the vitamin are without effect (J).

Different combinations of the members of the vitamin B complex in other experiments elicited no better response. In addition pyridoxal, and pyridoxamine were added to the vitamin group and these combined with purines, pyrimidines, and 14 amino acids to no avail (table 6, 7). Ten times the customary amounts of folic acid, pyridoxine, and B_{12} were found to be ineffectual as compared with the influence of the three basal vitamins (table 9: B, G).

D. Amino Acids, Purines, and Pyrimidines. Fourteen amino acids and 4 purines and pyrimidines were grouped together in the combinations and concentrations indicated in table 6. It was thought that in such a relatively complete medium the organism would have ample opportunity to develop well. The constitution of the medium may be noted in table 7 as well as the results.

TABLE 5

Influence of various members of the vitamin B complex on the proteolytic activity of the test organism

		Culture Designation										
		A	B	C	D	E	F	G	H	I	J	K
Constituents/100 ml of medium												
Vitamin-free casein	0.5 g											
Dextrose	0.1 g	x	x	x	x	x	x	x	x	x	x	x
Salt Mixture	0.5 ml											
Sorensen Buffer pH 7.0												
RO_2 = 47												
Thiamin	100 µg	x										
Niacin	100 µg	x	x	x	x	x	x	x	x	x	3x	
Biotin	4 µg											
Riboflavin	100 µg		x							x	3x	x
Ca. Pantothenate	100 µg			x						x	3x	x
Pyridoxine	100 µg				x					x	3x	x
Inositol	100 µg					x				x	3x	x
Folic Acid	100 µg						x			x	3x	x
Vitamin B_{12}	10 mµg							x		x	3x	x
P.A.B.A.	100 µg								x	x	3x	x
Formol titration difference after 40 days incubation		0.25	0.15	0.33	0.13	-0.03	0.11	0.14	-0.06	0.03	0.13	0.01

TABLE 6

Composition of the medium employed to test the influence of vitamins, purines, pyrimidines, and amino acids on the proteolytic activity of the test organism

Constituents/100 ml of medium	
Basal	
Vitamin-free casein	0.5 g
Salt Mixture	0.6 ml
Dextrose	0.1 g
Thiamin	100 µg
Biotin	4 µg
Niacin	100 µg
Sorensen Buffer pH 7.0	
RO_2 = 47	
Vitamin Supplements	
Riboflavin	100 µg
Ca. Pantothenate	100 µg
Pyridoxine	100 µg
Pyridoxal	100 µg
Pyridoxamine	100 µg
Inositol	100 µg
Folic Acid	100 µg
P.A.B.A.	100 µg
Vitamin B_{12}	10 mµg
Purines, Pyrimidines	
Adenine Sulfate	1000 µg
Guanine Hydrochloride	1000 µg
Uracil	1000 µg
Xanthine	1000 µg
Amino Acid Supplements	
L-Glutamic Acid	0.110 g
L-Cystine	0.002 g
L-Tryptophane	0.006 g
DL-Methionine	0.015 g
L-Tyrosine	0.026 g
L-Lysine	0.030 g
DL-Alanine	0.010 g
L-Aspartic Acid	0.020 g
DL-Valine	0.026 g
L-Histidine	0.010 g
L-Arginine	0.019 g
L-Proline	0.042 g
DL-Threonine	0.023 g
DL-Leucine	0.075 g

TABLE 7

Influence of vitamins, purines, pyrimidines, and amino acids on the proteolytic activity of the test organism

	Culture Designation			
	A	B	C	D
Constituents as Indicated in Table 6				
Basal	x	x	x	x
Vitamin Supplements		x	x	x
Purines, Pyrimidines			x	x
Amino Acids				x
Formol titration difference after 40 days incubation	0.22	-.04	-0.03	-0.03

It is apparent that in a 0.5 per cent concentration of casein, the amino acids, purines and pyrimidines were ineffectual. Other experiments using 1, 2.5, and 5 per cent of casein in combination with the amino acid and purine, pyrimidine supplements showed possible indications of increased proteolytic activity but the results were too variable to permit the forming of a definite opinion.

E. Dipeptides. Simmonds and Pruton (1949) reported on a prolineless mutant strain of E. coli which grew better with a proline peptide than with proline alone. It was thought desirable to test the effects of dipeptides on the proteolytic activity of the thermophilic organism. The medium was prepared as indicated in table 8 and the results show that the peptides were without influence and actually appeared to inhibit activity.

TABLE 8

Influence of peptides on the proteolytic activity of the test organism

		Culture Designation												
		A	B	C	D	E	F	G	H	I	J	K	L	M
Constituents/100 ml of medium														
Basal Medium														
Vitamin-free Casein	2.5 g													
Salt Mixture	0.5 ml													
Dextrose	0.1 g	x	x	x	x	x	x	x	x	x	x	x	x	x
Thiamin	100 µg			massive inoculum										
Niacin	100 µg													
Biotin	4 µg													
Sorensen Buffer pH 7.0														
RO_2 = 47														
Peptide Supplements														
Chloroacetyl-L-Tyrosine	1000 µg				x									x
DL-Leucyl-Glycine	1000 µg					x						x		x
L-Leucyl-Glycine	1000 µg						x						2x	x
Glycl-L-Leucine	1000 µg							x						x
DL-Alanyl-Glycine	1000 µg								x					x
D-Leucyl-L-Tyrosine	1000 µg									x		x		x
DL-Leucyl-Glycyl-Glycine	1000 µg										x	x		x
Formol titration difference after 21 days incubation		2.08	2.01	2.08	1.70	1.73	1.71	1.80	1.10	1.19	1.11	1.29	1.56	1.51

F. Peptone and Amino Acid Mixtures. Since the simple amino acids and the dipeptides were tested for their influence on the proteolytic activity of the thermophilic bacterium, it was thought advisable to complete a study of the effect of the nitrogen series of compounds by testing the influence of peptones as well as complete mixtures of amino acids. This has been done in the experiment listed under table 9. The composition of the peptone and the amino acid mixture supplements used in the experiment were as follows:

Peptone Supplements/100 ml of Media

(1)	Peptone (Bacto	7 µg
(2)	Protease Peptone No. 3 (Bacto)	7 µg
(3)	Tryptone (Bacto)	7 µg
(4)	Phytone (B.B.L.)	7 µg
(5)	Thiotone (B.B.L.)	7 µg
(6)	Neopeptone (Bacto)	7 µg
(7)	Protease Peptone (Bacto)	7 µg

Peptone and Amino Acid Supplements /100 ml of Media

(1)	Casitone (Bacto)	7 µg
(2)	Trypticase (B.B.L.)	7 µg
(3)	Casamino Acids (Bacto)	7 µg
(4)	Parenamine (Stearns)	7 µg

This experiment also tested the influence of various buffer systems, the composition of which were described in the section, Materials and Methods under the heading, Buffer Mixtures. Culture I was inoculated with organisms grown on trypticase yeast extract agar slants whereas all the other cultures, with an inoculum grown on casitone yeast extract agar slants.

TABLE 9

Influence of the basal constituents, peptone, amino acid and buffer mixtures on the proteolytic activity of the test organism

	Culture Designation												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Constituents/100 ml of Medium													
Vitamin-free casein 2.5 g Sorensen Phos. Buff. pH 7.0 RO_2 = 47	x	x	x	x	x	x	x	x	x	x	x	x	x no buffer
Salt Mixture 0.5 ml			x			x							
Dextrose 0.1 g				x		x							
Thiamin 100 µg Niacin 100 µg Biotin 4 µg					x	x							
Additional Vitamins													
Folic Acid 1000 µg Pyridoxine 1000 µg Vitamin B_{12} 10 mµg							x						
Peptone Supplements (see text)								x					
Peptone and Amino Acid Supplements (see text)										x			
Sorensen Citrate Buff. pH 6.6											x		
Prideaux-Ward Buffer pH 6.75												x	
Formol titration difference after 21 days incubation	0.11	0.01	-0.01	0.21	1.88	2.15	0.19	0.18	0.61	0.52	0.12*	0.26	0.18

* no growth

The composition of the media and the results obtained are found in table 9. Cultures A and B were the controls and showed a formol titration difference of 0.11 ml and 0.01 ml of 0.05 N NaOH respectively. When contrasted with these values, Culture H with 0.18 ml shows that the peptone mixture had no value with respect to the activity. Culture J with a value of 0.52 ml indicates that the peptone-amino acid mixtures favorably affected the proteolytic activity of the thermophilic organism. Some observations bearing on the factors studied in this experiment have already been reported under special headings so the results will be only briefly recapitulated. Culture I with a formol titration difference of 0.61 ml demonstrates the influence of a different inoculum source when compared with control Cultures A and B. Culture C proved the basal salt mixture to be of no value. Culture D with the dextrose gave a slightly improved activity. The three basal vitamins, thiamin, niacin, and biotin, demonstrated a considerably increased activity in Culture E with 1.08 ml as compared with Controls A and B. Culture F with all the basal constituents gave maximal activity; 2.15 ml Culture G with additional amounts of vitamins proved of no value. The Sorensen citrate buffer, Culture K, showed no activity nor did it even permit growth of the organism, whereas the Prideaux-Ward buffer, Culture L, showed some activity. Culture M, with no buffer present showed little or no evidence of activity. In tables 10, 11, and 12 are included all the data to accompany this experiment. Similar data for all the other experimental work have likewise been accumulated but have been omitted due to the voluminous nature of the material. Only the formol titration differences have been recorded.

TABLE 10

Formol titration data obtained from the experiment* on the influence of the basal constituents, peptone, amino acid, and buffer mixtures on the proteolytic activity of the test organism

Culture Designation	Formol Titrations					
	Controls			Cultures		
	Days of Incubation					
	0	10	21	0	10	21
	Ml of 0.05 N NaOH					
A	1.03	1.06	1.37	1.09	1.09	1.48
B	1.11	1.12	1.41	1.09	1.16	1.42
C	1.00	1.06	1.45	1.02	1.12	1.44
D	1.01	1.10	1.36	1.02	1.25	1.57
E	1.03	1.08	1.24	1.07	1.55	3.13
F	1.01	1.09	1.39	1.01	2.02	3.54
G	1.02	1.12	1.26	0.99	1.08	1.45
H	1.08	1.04	1.28	1.04	1.12	1.46
I	1.09	1.13	1.21	1.08	1.13	1.82
J	0.99	1.03	1.19	1.00	1.16	1.71
K	0.64	0.61	0.61	no evidence of growth		
L	0.81	0.73	0.64	0.80	0.67	0.90
M	0.64	0.64	0.44	0.65	0.53	0.62

*Culture conditions or supplements are those described in table 9.

TABLE 11

Formol titration difference and activity units data from the experiment* on the influence of the basal constituents peptone, amino acid, and buffer mixtures on the proteolytic activity of the test organism

	Formol Titration Difference			Activity Units	
	Days of Incubation				
	0	10	21	10	21
	Ml of 0.05 N NaOH				
Culture Designation					
A	0.06	0.04	0.11	0.004	0.005
B	-0.02	0.04	0.01	0.004	0.0005
C	0.02	0.06	-0.01	0.006	-0.0005
D	0.01	0.15	0.21	0.015	0.01
E	0.04	0.47	1.88	0.047	0.09
F	0.00	0.93	2.15	0.093	0.102
G	-0.03	-0.09	0.19	-0.009	0.01
H	-0.02	0.06	0.18	0.006	0.009
I	-0.01	0.00	0.61	0.00	0.029
J	0.01	0.13	0.52	0.013	0.026
K	no evidence of growth			no evidence of growth	
L	-0.01	-0.06	0.26	0.006	0.012
M	0.01	-0.01	0.18	-0.001	0.009

*Culture conditions or supplements are those described in table 9.

TABLE 12

pH data obtained from the experiment* on the influence of the basal constituents, peptone, amino acid, and buffer mixtures on the proteolytic activity of the test organism

	Controls		Cultures	
	Days of Incubation			
	0	21	0	21
	pH			
Culture Designation				
A	6.82	6.90	6.75	6.63
B	6.82	6.90	6.72	6.74
C	6.72	6.80	6.80	6.73
D	6.72	6.79	6.72	6.80
E	6.72	6.86	6.80	6.81
F	6.72	6.72	6.75	6.70
G	6.72	6.72	6.72	6.70
H	6.72	6.80	6.72	6.79
I	6.72	6.79	6.70	6.73
J	6.74	6.79	6.72	6.78
K	6.60	6.75	6.60	6.70
L	6.72	6.80	6.75	6.70
M	6.70	6.80	6.65	6.70

*Culture conditions or supplements are those described in table 9.

C. A Comparison of Acid, Enzymatic, and Bacterial Digestions of 2.5 per cent Casein. Concurrently with the previous experiment, a study was undertaken to compare the proteolytic activity of the experimental organism as previously determined, with the enzymatic and acid digestion of casein. A sufficient amount of the stock casein solution remaining from the previous experiment (table 9) was now employed in the acid-enzymatic digestion experiment which was designed as described in table 13. The formol titration data are detailed in table 14.

TABLE 13

The comparison of the acid hydrolysis and pancreatic digestion of a 2.5 per cent casein solution

	Digestion Flask Designation				
	A	B	C	D	E
Constituents / 100 ml of solution					
Vitamin-free Casein 2.5 g Sorensen Phosphate Buffer pH 8.4	x	x	x	x	x
Pancreatin Solution - 10 ml	100 mg	200 mg	500 mg	HCl	
Controls Vitamin-free casein 2.5 g Sorensen Phos. Buff. pH 8.4 Pancreatin Solution - 10 ml (inactivated)	o	o	o		

TABLE 14

Formol titration data obtained from the experiment* on the comparison of the acid hydrolysis and pancreatic digestion of a 2.5 per cent casein solution

	FORMOL TITRATIONS									
	Controls					Enzymes				
	Days of Incubation									
	0	1	3	9	15	0	1	3	9	15
	Ml of 0.05 N NaOH									
Digest. Flask Desig.										
A	1.00	1.00	1.25	1.10	1.10	1.18	2.05	2.30	2.45	2.44
B	1.12	1.10	1.51	1.24	1.06	1.17	1.97	2.56	2.55	2.44
C	1.01	1.06	1.37	1.27	1.05	1.44	2.25	2.71	3.02	2.80
D									3.59	3.92
E	0.84	0.96	1.37	1.13	1.06					

Formol titration differences and activity units corrected data (110 ml to 100 ml volume)

	Ml of 0.05 N NaOH								
	Formol Tit. Difference					Activity Units			
	Days of Incubation								
	0	1	3	9	15	1	3	9	15
Digest. Flask Desig.									
A	0.20	1.16	1.16	1.48	1.58	1.16	0.38	0.17	0.106
B	0.06	0.98	1.57	1.44	1.52	0.96	0.45	0.17	0.10
C	0.47	1.31	1.48	1.92	1.92	1.31	0.50	0.21	0.129

*Digestion conditions are those described in table 13.

An analysis of the vitamin-free casein used in the experiment is furnished by the Nutritional Biochemicals Corporation as follows:

Total Nitrogen, moisture free basis = 15%
Total Moisture, maximum = 8%

A 2.5% casein solution contains 2.5 g casein /100 ml.
The water content will therefore be 2.5 x 0.08 = 0.2 g H_2O.
Thus 2.5 - 0.2 = 2.3 g protein in 100 ml of 2.5% casein solution.
Now 2.3 x 0.15 = 0.345 g nitrogen present in 100 ml of 2.5% casein solution.
This is equivalent to 0.00345 g nitrogen /ml.
The atomic weight of nitrogen is 14.0 g, and a 0.05 N solution of nitrogen will contain 0.7 mg nitrogen/ml. In the formol titration one equivalent of an amino acid will be neutralized by one equivalent of alkali.
Since 0.7 mg nitrogen = 1 ml of 0.05 N NaOH therefore,
Theoretical total nitrogen in terms of NaOH =

$$\frac{0.00345}{0.0007} = 4.93 \text{ ml of } 0.05 \text{ N NaOH}$$

In other words if all the nitrogen contained in a 2.5 per cent solution of casein were made available in the formol titration, then 4.93 ml of 0.05 N NaOH would be required in the titration. Actually of course, the true value will be somewhat less than this since some of the nitrogen is in a form incapable of being titrated in the formol titration. Nevertheless this value represents a maximum limit the use of which enables the determination of the degree of hydrolysis.

If we refer to the formal titration differences in tables 14 and 11 respectively, we may list the results of the different digestions in terms of 0.05 N NaOH as follows:

Total nitrogen content of a 2.5% casein solution(1 ml)	4.93 ml of 0.05 NaOH
Acid hydrolysis of 2.5% casein solution (1 ml)	
14-D	3.90 ml " " "
Pancreatic digestion of 2.5% casein solution (1 ml)	
14-A, B (100 mg, 200 mg)	1.55 ml " " "
14-C (500 mg)	1.92 ml " " "
Bacterial digestion of 2.5% casein solution (1 ml)	
11-E	1.88 ml " " "
11-F	2.15 ml " " "
11-I	0.61 ml " " "

Percentage hydrolysis from data

We can now calculate the percentage hydrolysis from the above data as follows:

$$\frac{\text{acid hydrolysis (14-D)}}{\text{theoretical total nitrogen}} = \frac{3.90}{4.93} = 79.0\%$$ [2]

$$\frac{\text{pancreatic digestion (14-A, B)}}{\text{acid hydrolysis}} = \frac{1.55}{3.90} = 39.9\%$$

$$\frac{\text{pancreatic digestion (14-D)}}{\text{acid hydrolysis}} = \frac{1.92}{3.90} = 49.2\%$$

$$\frac{\text{bacterial digestion (11-F)}}{\text{acid hydrolysis}} = \frac{1.88}{3.90} = 48.0\%$$

$$\frac{\text{bacterial digestion (11-F)}}{\text{acid hydrolysis}} = \frac{2.15}{3.90} = 55.1\%$$

$$\frac{\text{bacterial digestion (11-I)}}{\text{acid hydrolysis}} = \frac{0.61}{3.90} = 15.7\%$$

Examination of the above data reveals that the method of acid hydrolysis of the casein solution was about 79 per cent effective in rendering the available nitrogen titrable by the formol titration.[2] The pancreatic digestion resulted in an approximate 40-50 per cent hydrolysis which compares favorably with the data from Sahyun (1948). In contrast it may be noted that the bacterial digestion ranged from approximately 16-55 per cent and thereby actually exceeding the hydrolysis induced by the commercial enzyme. These hydrolysis values represent a lower range than is probably consistent with the actual conditions. This is so because the acid hydrolysis converted relatively all the nitrogen present

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[2] This low recovery of the nitrogen was undoubtedly due to the fact that the casein was filtered through the Mandler filter for purposes of sterilization. Microkjeldahl determinations of such filtrates showed a 15-30 per cent retentivity of nitrogen by the filter depending upon the concentration of the casein filtered.

in the casein solution into titrable nitrogen. On the other hand, the enzymatic and bacterial digestions undoubtedly converted some fraction of the casein nitrogen into a form which still was not wholly titrable as for example 1 out of 2 nitrogen atoms in tryptophane, or 2 out of 3 nitrogen atoms in histidine (Melnick and Oser (1949). Nevertheless this treatment of the data affords some means of approximating the percentage of hydrolysis accomplished by the bacterium.

The implications of the bacterial digestion values may be further examined. For example, Culture 11-F shows a casein hydrolysis equal to approximately 55 per cent. This does not imply that 45 per cent of the original protein remained chemically intact when subjected to the action of the protease system of the test organism. The 55 per cent hydrolysis figure indicates only that 55 out of 100 percent available nitrogen in the 2.5 per cent casein solution was rendered chemically susceptible to the formol titration. A property which this nitrogen fraction did not possess prior to the bacterial action, by virtue of its being bound up in its original complex protein form. Further it is interesting to observe that although only 55 per cent of the total protein nitrogen in Culture 11-F had been reduced to such simple substances as amino acids which were capable of being titrated in the formol titration, yet fully 100 per cent of the original casein was now converted into simpler nitrogenous substances. This was readily shown by the heavy precipitate caused by the addition of acid to the control and the lack of any precipitate in the culture when subjected to the same treatment. Presumably all the protein in the culture had been degraded by bacterial action to the degree that it was no longer precipitable by acid.

This data has been plotted in figure 2. The differences in the proteolytic activity of the pancreatin and the thermophilic organism are readily discernable. The pancreatin acts immediately on the casein substrate and considerable hydrolytic changes are affected in the first day of incubation. The bacterial digestion proceeds at a much slower pace. The bacterial population must first build up, then the cells apparently die and release their protease to the medium. The enzyme then attacks the substrate and achieves greater hydrolytic optima, but in longer periods of time.

III Biological Factors

A. Nature of the Organism. The test organism (N.C.A. 1603) has been classified by Gordon and Smith (1949) as Bacillus stearothermophilus Donk (Emend). This group has been characterized as growing at 65 C, hydrolyzing starch, incapable of growth in 5 per cent NaCl broth, and failure to produce acetylmethylcarbinol. In addition most of their strains reduced nitrate to nitrite and were capable of hydrolysing gelatin.

B. Different Types and Concentrations of Inocula. It was thought that more active inocula might be obtained by growing the organism on different media containing varying ingredients and even including casein in one instance. When tested, these inocula gave variable results from which no definite conclusions could be drawn. The employment of massive inocula in culture flasks also permitted of no definite conclusions because of the variable results obtained.

C. Relation of Bacterial Numbers to Proteolytic Activity. Results obtained from attempts to gather information regarding population development of this organism when it was cultivated in casein media, were

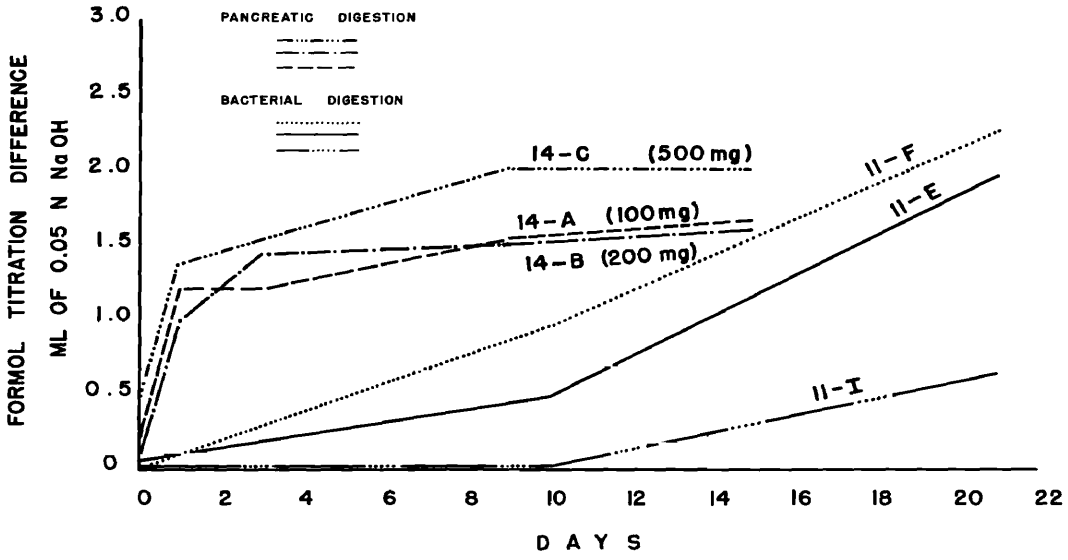

Figure 2. A comparison of the bacterial and pancreatic digestion of a 2.5 per cent casein medium

not very satisfactory. It was considered desirable to correlate viable and total counts with proteolytic activity and thereby gain a more complete picture of the process. Insofar as total counts were concerned, these were found to be quite difficult of accomplishment at times due to the production of "ghost" forms of the organism which refused to take the stain (Gaughran 1947). Consequently viable counts were found upon occasion to exceed total counts. This was undoubtedly due to the rapid autolysis of the organism with subsequent production of these "ghost" forms which were missed in the microscopic counts.

The innate sensitivity of the organism to an altered environment rendered the accomplishment of accurate viable counts difficult. Identical inocula were introduced into a series of different media, and immediately thereafter aliquots were withdrawn and plated out. Invariably the viable counts were not always identical for all culture flasks. At times the variation would range from 10^4 to 10^6 per ml. Also in plating out dilutions from a single culture flask it was difficult to get satisfactory counts representing the multiple dilutions. Indeed in some instances it was difficult to get satisfactory duplicate plates. This type of sensitivity or irregularity is discussed in the paper by Hansen (1933).

In view of these discordant results it is best to dwell in generalities with respect to the viable counts obtained in this problem. The inocula which were introduced into the culture flasks usually fell below 10^6 per ml, since it was considered of advantage to employ minimal amounts of the organism, just sufficient to initiate growth in the culture. There were innumerable instances of bacterial multiplication in the media, maximal viable counts never being higher than 6.0×10^7

per ml. There was no strict correlation between counts and proteolytic activity. For example, a certain bacterial count would be responsible for a high proteolytic activity, which could not be approached by another bacterial count that was 2 to 3 times higher than the first. It may be said however that good proteolytic activity was generally accompanied by high bacterial counts. Further, activity was usually preceded by the development of the bacterial population.

SUMMARY AND CONCLUSIONS

The physical, chemical, and biological factors which influence the proteolytic activity of an obligate, thermophilic bacterium (N.C.A. 1503) on a casein medium have been studied. The test organism was found to be incapable of growing at a temperature of 45 C in a casein medium. On the other hand it grew well in a similar medium at 55 C and exhibited proteolytic activity which was determined by the formol titration. The period of incubation was variable and found to be dependent upon the oxygen tension of the medium, the concentration of the casein employed, the constitution of the medium, and the condition of the inoculum. Optimal proteolytic activity was found to occur at pH 6.4-6.8 and 7.3-7.6.

The oxygen tension of the medium was found to have a marked influence on the proteolytic activity of the organism. A formula describing the oxygen tension of the medium was derived in which the oxygen tension (RO_2) was defined as varying directly with the surface area of the medium and inversely as the depth of the medium. An RO_2 equal to 74 was found to supply an optimal condition of oxygen tension, but an RO_2 equal to 47 was used in the course of the work due to the limitations of incubator space. The oxygen tension was also found to vary critically with the pH of the medium.

Proteolysis occurred in the Sorensen phosphate buffer at pH 7.0. This buffer system was employed throughout the course of the experiments. The Clark-Lubs buffer system at pH 9.0 and the Prideaux-Ward buffer

system at pH 7.0 also proved satisfactory when tested. The organism failed to develop in the Sorensen citrate buffer system at pH 7.0, however.

Proteolytic activity in a 0.5 per cent casein medium generally occurred within 4-6 weeks. It was found that this incubation period could be reduced to 2-3 weeks by increasing the casein concentration of the medium to 2.5 per cent. Under identical conditions of experiment, it was also noted that the use of two different lots of casein resulted in dissimilar proteolytic activities.

The casein medium was supplemented with a number of substances to determine the influence of these on the proteolytic activity of the test organism. It was found that the addition of 0.1 per cent dextrose generally stimulated the proteolytic activity of the thermophil. The salt mixture provided no stimulation at all when added to the casein substrate. When the three vitamins thiamin (1 µg/ml), niacin (1 µg/ml), and biotin (0.04 µg/ml) were added individually to the casein substrate, only thiamin was found to influence the proteolytic activity of the organism. When the three vitamins were added collectively to the casein substrate, the activity was much greater than the individual response of any of the vitamins. Maximal activity was obtained in a medium consisting of casein, dextrose, salt mixture, thiamin, niacin, and biotin.

When calcium and magnesium salts were added to the casein medium in a M/250 concentration at pH 7.0, it was found that the proteolytic activity was not influenced. The members of the vitamin B complex when added in 1 µg/ml amounts individually or in combinations to the casein medium, proved to be non-stimulatory. The addition of a series of 6 dipeptides and 1 tripeptide in 10 µg/ml amounts was likewise found to

be without influence on the proteolytic activity of the organism. A mixture of 7 peptones, each in 0.07 µg/ml amounts were added to the casein medium and proved ineffectual, but a combination of 2 peptones and 2 amino acid mixtures in the same concentration stimulated the proteolytic activity.

The bacterial digestion of the 2.5 per cent casein medium was compared with the pancreatic digestion and the acid hydrolysis of the same medium. As determined by the formol titration, the acid hydrolysis was most effective, resulting in 79 per cent hydrolysis of the casein. Bacterial digestion amounted to 55 per cent hydrolysis, and the pancreatic digestion achieved 50 per cent hydrolysis of the protein. On the other hand, the pancreatic digestion was more rapid than the bacterial digestion. The test organism altered the nature of the casein in such a manner that it was no longer capable of being precipitated by the addition of acid.

The thermophilic organism was found to exhibit an extremely innate sensitivity to an altered environment thereby causing total and viable counts to be difficult of accomplishment. Generally it was found that proteolytic activity was associated with the development of a large bacterial population.

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