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Proteolytic enzyme system of Streptococcus lactis

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NOTE TO USERS

PROTEOLYTIC ENZYME SYSTEM OF *STREPTOCOCCUS LACTIS*

by

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INTRODUCTION

Although *Streptococcus lactis* long has been recognized primarily as being saccharolytic, the results of various investigations have shown that many strains of this organism are capable of bringing about protein degradation when grown in milk. The breakdown of protein by microbial enzymes of the lactic acid bacteria as found in dairy products such as cheese and fermented milks may be important for several reasons.

In cheese ripening the breakdown of protein represents one of the most important changes. The protein hydrolysis changes the tough rubbery curd of young cheese into material with a smooth, waxy consistency. It generally is accepted that the development of cheese flavor is associated with concurrent proteolytic and lipolytic changes.

The hydrolysis of cheese protein has been attributed in part to the action of the natural proteinase in milk, to the rennet proteinases and to the proteinases of microbial origin, although different investigators have disagreed upon the relative importance of the various proteinases in the breakdown of cheese protein.

Since organisms such as *S. lactis* and *L. casei* are predominant in cheese during manufacturing and ripening, these

organisms are believed to be sources of microbial proteinases which contribute to the breakdown of cheese protein. Although many investigators are of the opinion that the proteolytic enzymes of S. lactis, which possibly may contribute to the breakdown of protein during the ripening process of cheese, are endocellular in nature and that their activity may have to wait upon death and autolysis of the cells, there is recently some indication in the literature that a substantial portion of the proteolysis caused by S. lactis when grown in milk can occur during the first 24 hours when autolysis is apparently negligible. Changes in milk protein then can be expected to take place in dairy products in which S. lactis is grown for a relatively short time. Proteolysis during the early phases of growth has received little or no attention, mainly because techniques commonly used, such as Kjeldahl analysis of acid-soluble nitrogen, did not show definite changes which could be associated with protein hydrolysis.

Little is known about the mode of action of the microbial enzymes that are believed to participate in the breakdown of milk protein. It appears from the literature that the proteolytic enzymes of microorganisms are exceedingly numerous, yet our information concerning them is very sparse as compared to the present knowledge of the animal enzymes. In recent years purification and crystallization of a few

proteinases important in industry and medicine has been achieved.

The present investigation was initiated to study the characteristics of the proteolytic enzyme system of S. lactis. Investigation of the breakdown of milk protein by microbial enzymes may be expected to yield information in regard to some problems important to the dairy industry, such as the ripening process of cheese and some of the changes that may occur in proteins in other dairy products.

STATEMENT OF PROBLEM

The purpose of this investigation was to study the factors involved in the production of the proteinases of S. lactis and some of the characteristics of this enzyme system in regard to the breakdown of milk proteins.

REVIEW OF LITERATURE

Proteolytic Activity of S. lactis and Cheese Extracts

S. lactis long has been recognized primarily as being saccharolytic. However, the results of various investigations have shown that many strains are capable of causing slight protein degradation in milk. Early studies concerning proteolysis by S. lactis in milk were reviewed by Hammer and Babel (34). In most of these studies proteolysis was determined by (a) Kjeldahl analysis of nitrogen soluble in acetic acid, trichloroacetic acid or phosphotungstic acid, (b) the determination of free amino acids by either the Van Slyke method or the formol titration, or (c) ammonia determinations. In most cases, milk inoculated with S. lactis was incubated for periods ranging from several days to months, with and without calcium carbonate. Commonly protein cleavage was greater when part or all of the acid was neutralized by calcium carbonate. No attempt will be made to present a detailed discussion of the early investigations since they have been reviewed by others(17,34,35,44, 60). However, attention will be given to more recent work on the microbial proteinases, particularly those of S. lactis.

Collins and Nelson (18) studied the proteolytic activity

of four strains of S. lactis. Grown in milk, this organism caused a rapid increase in trichloroacetic acid-soluble nitrogen during the first day or two, followed by a gradual but much smaller increase throughout the 15 day test period until approximately 15 per cent of the total nitrogen was in soluble form. The magnitude of the increase varied slightly with different strains of S. lactis.

Zimmerman (60) reported that a cell-free extract of S. lactis, using either casein or hemoglobin as substrate, did not show proteolytic activity. The same extract caused hydrolysis of glycyl-L-leucine and DL-alanylglycine. Measurements of hydrolysis of glycyl-L-leucine indicated an optimum at pH 7.6 to 7.8. For the hydrolysis of DL-alanylglycine an optimum was indicated at about pH 8.0. Manganous ions increased the rate of hydrolysis of glycyl-L-leucine. No appreciable difference in the rate of ripening was found when the cell-free extract was added to cheddar cheese.

Amundstad (2) reported that cell-free extract of each of two strains of S. lactis grown in broth medium showed optimum activity at pH 6.6 to 6.8 in both peptone broth and caseinate solutions, as determined by the increase in acid-soluble nitrogen and amino nitrogen. A cell-free extract of S. cremoris showed optimum activity at pH 6.3 to 6.5 and proved to have greater proteolytic activity than the extract prepared from S. lactis. Morgan and Nelson (45) studied the

distribution of ten amino acids in tungstic and lactic acid filtrates of milk after incubation with S. lactis for 15 days at 21° C. Microbiological assays for valine, leucine, isoleucine, threonine, arginine, methionine, histidine, tryptophan, tyrosine and phenylalanine revealed marked increases over the values for the filtrates from the uninoculated controls. Active strains of S. lactis were able to effect a marked increase in leucine and phenylalanine during the first three days of incubation with a slower increase through the fourteenth day.

Baribo and Foster (8) determined some of the characteristics of a cell-free extract of one strain of S. lactis grown in carrot-liver extract broth. Proteolysis was determined by Kjeldahl analysis of nitrogen soluble in trichloracetic acid; sodium caseinate was used as substrate. The cell-free extract was most active at 40 to 42° C. and at pH values near neutrality; evidence of a second optimum was observed at pH 5.0 to 5.5. The proteolytic activity was relatively stable over a considerable pH range (3.4 to 8.6) and was activated by reducing agents. Metallic ions either had no effect on the activity of the extract or were inhibitory.

In recent years more attention has been given to the proteinases of microorganisms that are predominant in cheese during manufacturing and ripening, including S. lactis and L. casei. The breakdown of cheese protein has been

attributed to the natural proteinases in milk, to the proteinases in rennet and to the proteinases of microbial origin, although different investigators have disagreed upon the relative importance of the various proteinases.

Peterson, Johnson and Price (49) showed the presence of proteolytic activity in a water extract of cheddar cheese. Proteolysis was determined by the increase in non-protein nitrogen in a digestion mixture with casein as substrate. The optimum pH for casein hydrolysis by the cheddar cheese proteinase system appeared to be 5.0; a secondary optimum occurred at pH 7.0 to 8.0. The proteolytic activity at pH 5.0 was enhanced in the presence of reducing agents, particularly cysteine. These workers (50) also reported on the proteinase content of raw and pasteurized milk cheddar cheese at intervals during the making and ripening period. The active proteinase in ripening cheddar cheese was considered to be largely of bacterial origin; only a small fraction of the total activity was contributed by the milk and the rennet. Pasteurized milk cheddar cheese was characterized by a lower content of cysteine-activated proteinase.

Baribo and Foster (8) reported that an extract of cheddar cheese showed maximum proteolytic activity against casein at pH 5.0 and at near neutrality. It was most active above 45° C., showed a second optimum between 11 and 18° C. and was stable over a considerable pH range. This

proteolytic system withstood heating at 60° C. (pH 5.1) for 30 minutes and was stimulated by reducing agents; metallic ions had no effect or were inhibitory.

Microbial Proteinases

A review of the literature on microbial proteases up to 1929 was presented by Oppenheimer (48). The early work on bacterial peptidases was reviewed by Berger, Johnson and Peterson (12). No attempt will be made to discuss the early investigations in detail. Instead, those investigations will be reported in which progress has been made in the study of the particular system isolated. It appears from the reviews of early work that numerous reports have appeared in the literature on the proteolytic enzymes of microorganisms, especially on those enzymes which are secreted into the medium by the living cell or are present as the result of autolysis. However, in most cases only a few data illustrating proteinase and/or peptidase activity are presented.

Berger, Johnson and Peterson (11) probably were the first ones to study systematically the bacterial peptidases. Autolyzed cell preparations were used in a study of the peptidases of Leuconostoc mesenteroides. Evidence was obtained that at least two dipeptidases and two tripeptide-splitting enzymes were present. Hydrolysis was activated by

a rather large number of metallic ions. The peptidases possessed two pH optima for activity, one at pH 5.0 to 6.0 and the other at pH 7.0 to 8.0. The authors observed rapid action on the D-forms of a variety of peptides; D-leucylglycylglycine was split about as fast as the enantiomorph. The same workers (12) presented an excellent survey of the peptidases present in cell-free extract of some common bacteria such as Escherichia coli, Bacillus megatherium, Proteus vulgaris, Ps. fluorescens, B. mesentericus (B. subtilis) Clostridium butylicum, Cl. sporogenes, Cl. acetohistolyticum, L. pentosus, Propionibacterium pentosaceum and Phytomonas tumefaciens. In most cases, optimum hydrolysis occurred at pH 8.0 to 9.0; the enzyme of L. pentosus and P. pentosaceum had optimum activity at pH 5.0 to 6.0.

Metal activation of peptidase preparations of P. tumefaciens, B. megatherium, and P. vulgaris was studied by Berger and Johnson (9). Leucylpeptidase of P. vulgaris was activated by manganous and magnesium ions. Hydrolysis of glycylglycine by peptidases of B. megatherium also was activated by these ions. Berger, Johnson and Bauman (10) studied the effect of D-peptidases from B. megatherium, L. mesenteroides and a few other organisms. All seemed to possess peptidases active against D-leucylglycine. Most of these preparations were activated by manganous or cobalt ions or with cysteine and these ions. Optimum pH for

D-peptidase of L. mesenteroides was found to be between pH 7.0 to 8.0.

Gorbach (29) reported on the proteolytic system of Caseococcus Gorini (S. liquefaciens). The hydrolysis of DL-leucylglycine showed optima at pH 4.8 and at 7.0 to 8.4. Later Dudani (20) determined some of the characteristics of peptidases present in a cell-free extract of S. liquefaciens. The cell-free extract was active against glycyl-L-leucine and DL-alanylglycine; the latter was hydrolyzed more readily. Two pH optima for peptidase activity against glycyl-L-leucine were found, one at pH 8.0 and the other at pH 5.0. Two pH optima for DL-alanylglycine also were observed, one at pH 6.9 and the other around pH 8.2. Hydrolysis at pH 8.2 was activated by manganous, cobalt and magnesium ions. Zimmerman (60) reported that a cell-free extract of S. cremoris showed optimum activity at pH 8.0 against both glycyl-L-leucine and DL-alanylglycine.

Tarnanen (56) reported that a cell-free extract of Bacterium casei ε (Lactobacillus helveticus) was active against casein, gelatin and peptone; the maximum activity was at pH 6.0 and 42° C. Ramon et al. (51) reported that B. subtilis is a producer of an active gelatinolytic enzyme. From culture filtrates of Cl. welchii, Bidwell and van Heyningen (14) have isolated a collagenase (K-toxin) which attacks only gelatin and collagen. The partially purified enzyme has its optimum activity at pH 6.8 to 7.5 and is free

of other toxins of the organism. The enzyme appeared to have clinical importance, since such culture filtrates injected into rabbit muscle disintegrated the collagen frame work. A similar collagenase was obtained by Kocholaty and Krejci (40) as an electrophoretically homogeneous protein from cultures of Cl. histolyticum. It was activated by iron and sulfhydryl groups. The enzyme hydrolyzes gelatin and clupein. Evans (25) has reported that extracellular collagenase is produced by 30 strains of Cl. welchii.

Gorini and Fromageot (30) have found that Micrococcus lysodeikticus produces a proteinase which causes strong liquefaction of gelatin and digestion of casein. It was inactive in the absence of calcium ions. Addition of phosphate, citrate or oxalate gave complete inactivation which was readily reversible by addition of calcium ions. Dudani (20) studied some of the characteristics of an extracellular proteinase of S. liquefaciens. It had its optimum activity at pH 7.4 for digestion of both casein and lactalbumin substrates. The proteolytic activity proved to be adaptive; casein was not proteolyzed to any great extent by the extracellular enzymes of S. liquefaciens unless casein was included in the medium used for growth of the organisms and elaboration of the enzymes.

In recent years a few workers have succeeded in the crystallization of microbial proteinases. Isolation and crystallization of a proteinase resembling papain and its

precursor has been reported by Elliott and coworkers (21,22, 23,24,46) from strains belonging to group A of the hemolytic streptococci. Grown under certain conditions some group A streptococci lost their type specific protein antigen (M-substance). This temporary loss resulted from proteolytic activity in the bacterial culture during the later stages of growth. The enzyme originated from a precursor by autocatalysis or by the action of trypsin. Excellent characterization was obtained by studying the reaction of the crystalline enzyme on a great number of synthetic substrates and derivatives. It proved to have a very broad side-chain specifity. Crewther and Lennox (19), using fractional precipitation with ethanol and ammonium sulfate, prepared needle-shaped crystals of proteinase from a culture fluid of Aspergillus oryzae. Güntelberg and Ottesen (33) prepared crystals containing the plakalbumin-forming enzyme from a culture fluid of B. subtilis by precipitation with acetone. This enzyme is able to convert egg albumin (needles) to plakalbumin (platelets) in which process two peptides are split off. From the reports reviewed here it is apparent that except for the proteinase isolated by Elliott and coworkers no adequate characterization of a microbial proteinase yet has been made.

Methods for Determining Proteolysis

A great number of methods are available for measuring the activity of proteolytic enzymes. Only those methods which were of most interest to the author in connection with this investigation will be mentioned. Kjeldahl analysis of nitrogen soluble in various fractions, the determination of free amino acids by either the Van Slyke method or the formol titration and ammonia determinations are some of the methods most frequently used. Digestion of milk, casein and hemoglobin by the enzymes, followed by colorimetric determination of tyrosine and tryptophan in a trichloroacetic acid filtrate of the substrate using Folin-Ciocalteau reagent (26) also has been frequently employed (3,4,7,21, 36,39,60). Methods involving titration of carboxyl- or amino groups have been used widely in the study of hydrolysis of peptides and derivatives. Methods for titrating carboxyl groups with ethanolic alkali were worked out by Foreman (27), Wilstätter and Waldschmidt-Leitz (59), and Grassmann and Heyde (31). Linderstrøm-Lang (43) reported on a titrimetric method for the determination of amino nitrogen, involving the titration of amino groups with ethanolic HCl, upon the addition of acetone, using naphtyl red as indicator. A sensitive photometric ninhydrin method for measuring proteolysis has been proposed by Schwartz and

Engel (52). Sensitive methods of determining proteolysis based upon the ability of native protein to bind dyes have been reported. Greif (32) used the dye bromsulfalein and Carroll (16) used anionic dyes such as Orange I.

EXPERIMENTAL METHODS

Designation and Sources of the Cultures and Their Propagation

S. lactis strains 18, 26 and 34 used in this study were from a group of 13 pure cultures isolated from different samples of raw cream and were selected on the basis of their ability to produce the greatest increase in non-protein nitrogen when grown in milk. The streptococci were identified as S. lactis by the characteristics given by Sherman (53). The stock cultures were transferred weekly and were carried in sterile litmus milk. Cultures were transferred every day for three transfers prior to each trial, using the culture medium employed in the experiment. Cultures were incubated at 32° C.

Preparation of Media

In the course of this investigation the following media were used for growing S. lactis.

Skim milk

Unless stated otherwise, fresh skim milk from the College Creamery was heated at 185° F. for 20 minutes to

destroy as many undesirable organisms as possible without subjecting the milk constituents to changes that might occur during sterilization.

Niven and Sherman's medium

The simplified amino acid medium of Niven and Sherman (47) was used. Stock solutions for preparing the synthetic medium were kept in brown bottles in a cold room at approximately 2° C. Toluene was added in a thin layer to all stock solutions as a preservative.

Vitamin-test casein medium A

The composition of this medium was:

Vitamin-test casein	20	g.
Glucose	6.5	g.
$K_2HPO_4 \cdot 3H_2O$	10	g.
Riboflavin	1	mg.
Calcium pantothenate	1	mg.
Nicotinic acid	1	mg.
Pyridoxine·HCl	1	mg.
Thiamine	1	mg.
Biotin	1	γ
Folic acid	10	γ
Vitamin B_{12}	20	γ
Distilled water to	1000	ml.

Twenty g. of vitamin-test casein (Nutritional Biochemicals) were suspended in 400 ml. 0.05 N NaOH and steamed for 10 minutes. An equal volume of distilled water was added and at this stage $K_2HPO_4 \cdot 3H_2O$ and glucose were added and the pH was adjusted to 6.8 to 7.0. The vitamins then were added, the volume was made up to 1 l. with distilled water and the medium was autoclaved for 13 minutes at 15 lb. pressure.

Vitamin-test casein medium B

The composition and preparation of this medium are the same as given for vitamin-test casein medium A, except that the vitamins were replaced by 10 g. yeast extract.

Casein medium

The composition and preparation of this medium are the same as given for vitamin-test casein medium A, except that the vitamin-test casein was replaced with an equal amount of technical casein.

Tryptone medium

The composition and preparation of this medium are the same as given for vitamin-test casein medium B, except that vitamin-test casein was replaced with an equal amount of Bacto tryptone.

Amundstad's medium

This medium was used by Amundstad (2) to grow S. lactis for the preparation of cell-free extracts. This medium has the following composition:

Peptone	20 g.
Glucose	10 g.
Lactose	10 g.
$MgSO_4$	1 g.
K_2HPO_4	2 g.
NaCl	2 g.
Yeast extract (infusion)	500 ml.
Water	455 ml.

In the preparation of the yeast infusion, 1 part cake yeast was boiled for 0.5 hour with 2 parts of water, followed by settling for 12 hours. The clear supernatant was used in the medium. This medium was adjusted to pH 6.8 to 7.0 and autoclaved for 13 minutes at 15 lb. pressure.

Tryptone glucose beef extract broth

The composition of this broth was as follows: 3 g. beef extract, 5 g. tryptone, 1 g. glucose and 1000 ml. distilled water. The medium was adjusted to pH 6.8 to 7.0 and autoclaved for 13 minutes at 15 lb. pressure.

Determination of Proteolysis by *S. lactis* Grown in Milk

Procedure for trials with and without controlled pH

Milk for the trials without controlled pH consisted of fresh skim milk which was heated at 185° F. for 20 minutes to destroy as many undesirable organisms as possible without subjecting the milk constituents to changes that might occur during sterilization.

Because of the absence of a rapid drop in pH in the cultures with maintained pH (5.5 to 8.5), milk for these samples and their controls was autoclaved for 12 minutes at 15 lb. pressure. Skim milk was chosen because the absence of fat facilitated accurate sampling and analysis. This milk (200 ml.) was pipetted into a sterile beaker covered by a double layer of sterile aluminum foil with holes (covered with sterile cotton) to fit the electrodes and stirrer of a Beckman model K automatic titrator placed in a 32° C. incubator. After addition of 0.1 per cent inoculum, the sample was kept at the desired pH by sodium hydroxide delivered from the automatic titrator. The accuracy of pH control was checked at regular intervals by determining the pH of the sample with a Beckman pH meter; deviations from the desired pH value were not more than 0.15 of a unit. Inoculated samples (200 ml.), in screw-top bottles, without

maintained pH were run at the same time without agitation as controls. Before inoculation, all samples were brought to the desired initial pH with sodium hydroxide or sulfuric acid. At certain intervals samples for analysis were withdrawn with sterile pipettes. Changes in volume were recorded carefully and necessary corrections were made in the controls with sterile distilled water.

Determination of nitrogen in skim milk fractions

Nitrogen fractions insoluble in trichloroacetic acid were precipitated by the method developed for cheese serum by Lane and Hammer (41), except that in this work a 5 g. sample was used, while Lane and Hammer used 1 ml. of cheese serum. Portions of the coagulated cultures were blended for 5 minutes in a sterile Waring blendor with a semi-micro attachment. To 5 g. of sample were added 40 ml. of distilled water and 5 ml. of a 20 per cent solution of trichloroacetic acid, after which the mixture in each beaker was stirred vigorously. After standing overnight in a refrigerator, the mixtures were filtered through paper and the precipitates were washed with a 2 per cent solution of trichloroacetic acid. All nitrogen determinations were made in duplicate by the Kjeldahl procedure. Digestion was carried out using 10 g. of sodium sulfate, 2 g. of copper sulfate and 20 ml. of concentrated sulfuric acid. After digestion was complete and the solution had cooled, 200 ml. of distilled water,

100 ml. of sodium hydroxide (480 g. of 76 per cent sodium hydroxide per 1.) and a few zinc pellets were added to each flask. Distillate was caught in 50 ml. 0.05 N hydrochloric acid containing 8 drops of methyl red-methylene blue indicator (38); back titrations were carried out using 0.05 N sodium hydroxide. For total nitrogen determinations, duplicate 5 g. portions were pipetted directly into 500 ml. Kjeldahl flasks. All results were calculated on the basis of 5 g. of original milk.

Determination of tyrosine and tryptophan

Proteolysis also was determined by the increase of the amino acids tyrosine and tryptophan in trichloroacetic acid filtrates of the milk samples as described on page 28, except that the acid was added directly to a 5 ml. portion of the milk after incubation. Results are calculated as increase of tyrosine per 5 ml. of original milk.

Determination of titratable acidity

Titratable acidities were run on 9-ml. samples using 5 drops of 2 per cent phenolphthalein as indicator and titrating with 0.1 N sodium hydroxide.

Bacterial count

Bacterial counts were made according to Standard Methods

for the Examination of Dairy Products (1), with incubation at 32° C.

Determination of pH

A Beckman model G glass electrode pH meter was used for all pH determinations.

Preparation of Cell-Free Extracts of S. lactis

Growing and harvesting of cells

For the preparation of cell-free extracts, S. lactis was grown in skim milk and also in broth media, which were dispensed in 6-l. quantities in pyrex carboys. The milk used for growing S. lactis was heated at 185° F. for 20 minutes; the broth media were autoclaved for 13 minutes at 15 lb. pressure. The media then were cooled rapidly to 32° C., adjusted to pH 6.8 to 7.0 and inoculated with 1 per cent of a 24-hour culture of S. lactis which had been transferred daily for three transfers, using the culture medium employed in the experiment. The inoculated media were incubated for 8 to 10 hours at 32° C., an incubation period which gave a satisfactory yield of young, metabolically active cells of demonstrated high proteolytic activity. During incubation the pH was kept at 6.8 to 7.0 by periodic addition of sodium hydroxide.

The cells from the broth media were collected in a

Sharples Super centrifuge operated at 30,000 r.p.m. They were washed twice with ice cold M/15 phosphate buffer (pH 7.0), recovered by centrifugation in a refrigerated centrifuge and stored in a refrigerator.

Following incubation, the milk was run through a Sharples Super centrifuge at 30,000 r.p.m. The material collected in the bowl of the centrifuge was cooled immediately to 2° C., mixed with a few grams of sterile sea sand and some sterile distilled water and ground in a chilled mortar until all material had gone into a fine suspension. Centrifugation at 4,000 r.p.m. for 30 minutes separated this material into distinct layers, the cells forming the top layer. The cells were removed with a spatula, and washed and stored as described above.

Estimating the yield of cells

A count was obtained by dispersing a certain weight of wet cells (usually 250 mg.) in a 100 ml. water blank. Appropriate dilutions were prepared and counts were made on T. G. E. M. agar; the plates were incubated at 32° C. for 48 hours.

Preparation of cell-free extract by freezing and thawing

Washed cells were frozen and thawed eight times in five days. The cells then were mixed with two parts of M/15 phosphate buffer (pH 7.0) to one part of cells and stored

overnight in a refrigerator. Cell debris was removed by centrifugation. The clear supernatant was stored in a freezer unit.

Preparation of cell-free extract by grinding

The method used for grinding cells was as described by Utter and Werkman (57). Powdered pyrex glass or alumina powder were combined in the proportions of two parts of powdered glass or alumina powder to one part of bacterial cell paste. M/15 phosphate buffer (pH 7.0) to give the consistency of a thick batter was added. The grinding was accomplished by passing the mixture of bacteria and grinding material between concentric glass cones. The inner cone was rotated by a motor. The inner joint and its length of tubing was filled with crushed ice. The extruded material was caught in a chilled Petri dish. The paste was extracted with 1.5 ml. distilled water for each gram of bacteria used. The debris was removed by centrifugation. The clear supernatant was stored in a freezer unit.

Preparation of cell-free extract by sonic vibration

It soon was apparent that more uniform extracts could be prepared using a Raytheon Magnetostriction oscillator. Cell-free extracts were prepared as follows: seven ml. M/15 phosphate buffer (pH 7.0) were added to 7 g. of wet cells and the mixture was stored overnight in a freezer

unit. Next day 7 ml. M/15 phosphate buffer (pH 7.0) were added to the frozen suspension. After thawing, the suspension was poured into the chilled cup of a Raytheon 9 kc. Magnetostriction oscillator. Disintegration was allowed to take place for 90 minutes, during which the cup was cooled by running ice cold water through the space between the two walls of the cup. Intact cells and cell debris were removed by centrifugation. The clear supernatant was removed with a medicine dropper. The debris was mixed with 7 ml. ice cold M/15 phosphate buffer (pH 7.0) and the supernatant collected after centrifugation was added to the first portion. The combined extracts were stored in a freezer unit and used within a few days.

Determination of Total Proteolytic Activity

Preparation of substrates

Skim milk and 2 per cent solutions of casein and lactalbumin were used as substrates. Pasteurized skim milk was brought to the desired pH and stored at 2° C. A 2 per cent solution of casein was prepared by suspending 4 g. of casein (Fischer Scientific Co.) in approximately 100 ml. distilled water, adjusting to pH 10.0 with sodium hydroxide and heating in a steamer for 10 to 15 minutes. After cooling, the solution was adjusted to the desired pH, followed by addition of 20 ml. of a 0.2 M composite buffer of that pH value,

and made up to volume with sterile distilled water. Two per cent solutions of α, β and γ casein were prepared as described for casein. A 2 per cent solution of lactalbumin (Supplee Research Co.) was prepared as described for casein, except that lactalbumin was used. Merthiolate was added to all substrates as a preservative at the rate of 1 mg. to 40 ml. substrate; this concentration had no adverse effect on the enzyme actions studied. All substrates were stored at 2° C.

Modification of a test for tyrosine and tryptophan

Hull (37) found the method of Anson (4), using a denatured hemoglobin solution as a substrate to measure proteolytic activity, most adaptable for measuring the hydrolysis of milk protein. In this method proteolysis is determined by the increase of the amino acids tyrosine and tryptophan in a trichloroacetic acid filtrate of the hemoglobin substrate by measuring the blue color in a photometer after addition of Folin-Ciocalteau reagent. When milk is substituted for the denatured hemoglobin solution, several changes have to be made in the reagents. The method of Anson (4) calls for a 0.3 N trichloroacetic acid solution to precipitate the protein hemoglobin. The concentration must be increased to 0.72 N in order to remove the milk proteins. In preparing the protein-free filtrate for addition of Folin-Ciocalteau reagent, it is advisable to use a 15 per cent sodium carbonate solution containing enough polyphosphate to

prevent precipitation of the calcium salts of milk during the addition of the carbonate. This solution can be prepared by dissolving 75 g. of anhydrous sodium carbonate and 10 g. of sodium tetraphosphate (Quadrafos) in distilled water and diluting the solution to 500 ml. after the material has gone into solution.

Determination of proteolysis

The digestion mixture, in sterile screw-top test tubes, consisted of 5 ml. skim milk, plus cell-free extract and sterile distilled water to make a total volume of 7 ml. The cell-free extract was added when the substrate had reached the temperature of incubation; incubation for the desired length of time was in a water bath at 37.5° C. At the end of the incubation period the enzyme action was stopped by adding 10 ml. of a 0.72 N trichloroacetic acid solution to the digestion mixture, while agitating the test tube to mix the milk and the acid. The tube was allowed to stand for 10 minutes before filtering through paper. One ml. of protein-free filtrate was added to a 50 ml. Erlenmeyer flask, followed by 4 ml. distilled water and 10 ml. of the sodium carbonate-quadrafos reagent. The contents of the flask were mixed thoroughly before 3 ml. of the Folin-Ciocalteau reagent were added under continuous shaking. Five minutes were allowed for the blue color to reach a maximum before reading was performed in a Klett-Summerson

photoelectric colorimeter, using a 645 mμ wave-length filter.

Since the substrate and cell-free extracts used commonly contained some color-producing products, as shown by this test, blanks were run on them by adding the required amount of cell-free extract to the substrate after the trichloroacetic acid was added. Readings of the blank were subtracted from the test determination. Previously prepared standard tyrosine curves, showing the colorimeter readings for various tyrosine concentrations, then were used to convert the sample reading into its tyrosine equivalent (Figure 1). The procedure used for color development of the standard solutions was the same as that employed for the milk samples. The tyrosine used in the standard solutions was L-tyrosine (Merck), recrystallized and dried. A sample was checked for purity by determining its nitrogen content by the Kjeldahl-Gunning-Arnold (6) method and 98.85 per cent of the calculated theoretical quantity of nitrogen was found.

In calculating the results, the tyrosine content is based upon its concentration in 1 ml. of filtrate used for the production of the blue color. It is recognized that these values represent not only free tyrosine and tryptophan but also some of the smaller peptides containing these amino acids in positions where they may react with the Folin-Ciocalteau reagent. The procedure followed for the casein and lactalbumin substrates was similar to the one used for

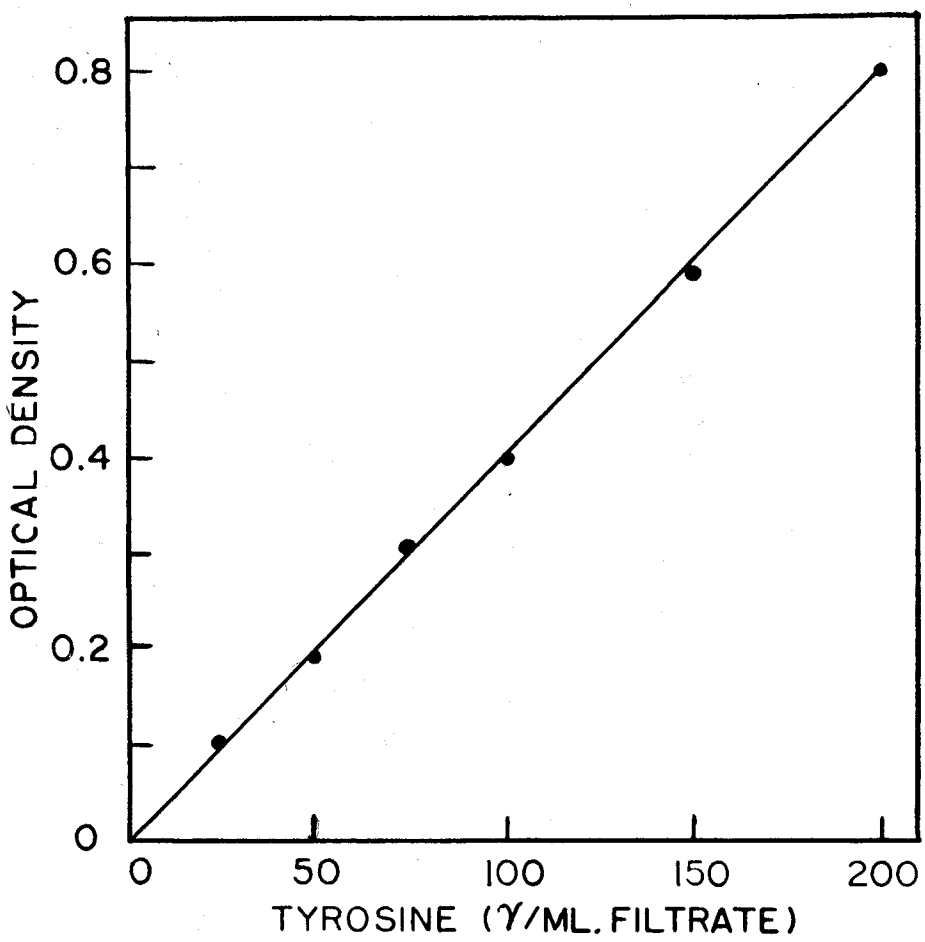

Figure 1. Standard Curve Relating Micrograms of Tyrosine to Color Produced by Folin-Ciocalteau Reagent.

milk, except that 0.6 N trichloroacetic acid was used, whereas in the case of milk 0.72 N acid was used.

Hydrolysis of Peptides

Preparation of substrates

M/30 solutions of glycyl-L-leucine, glycyl-L-tyrosine, glycylglycine, glycylglycylglycine and M/15 solutions of DL-alanylglycine, DL-leucylglycine and DL-leucylglycylglycine were used as substrates. The substrates in 25 ml. quantities were prepared by dissolving the required amount of peptide in approximately 15 ml. distilled water and 7.5 ml. of a 0.05 M composite buffer of the desired pH. The solution then was brought to the desired pH with dilute sodium hydroxide or sulfuric acid and made up to volume with distilled water. Merthiolate was added to all substrates at the rate of 0.6 mg. per 25 ml. substrate as a preservative; this concentration had no adverse effect on the activity of the peptidases. All substrates were stored at 2° C.

Determination of hydrolysis by cell-free extracts of S. lactis

The reaction mixture, in screw-top test tubes, consisted of 3 ml. of substrate plus cell-free extract (usually 0.2 ml.). The cell-free extract was added when the substrate

had reached the temperature of incubation; incubation for the desired length of time (usually 1 hour) was in a water bath at 37.5° C. Hydrolysis of the peptides by the cell-free extracts was determined by titration of the carboxyl groups as described by Grassmann and Heyde (31). Both at the beginning and end of the incubation period, 1 ml. of the reaction mixture was titrated with 0.05 N ethanolic potassium hydroxide, using a 0.1 per cent ethanolic solution of thymolphthalein as indicator. Immediately after addition of cell-free extract to the substrate, 1 ml. of the mixture was transferred to a test tube, followed by addition of 5 drops of the indicator solution. A solution of 0.05 N ethanolic potassium hydroxide then was run in from a microburette until the contents turned blue. At this stage absolute ethanol (9 times the volume of the mixture to be titrated) was added, which was accompanied by a disappearance of the blue color. More ethanolic potassium hydroxide was added until the endpoint was reached as represented by the bluish color of a 0.0025 M solution of $CuCl_2$ with excess ammonia. The titration was carried out in 20 x 175 mm. test tubes. This had the advantage that the color change when approaching the endpoint in the titration is more gradual, proceeding from top to bottom; the color may be observed through a depth of solution, which served to make slight color differences more detectable.

Per cent hydrolysis was calculated, after subtracting

the initial titration value from the value obtained at the end of the incubation period, from the number of moles of substrate present at the beginning of the experiment and the alkali used. The results are expressed as per cent hydrolysis of one linkage; in case of the DL-form, per cent hydrolysis of one linkage of one optical component (L-) was calculated.

Protein nitrogen determination by the biuret test

In the course of purification of the cell-free extract, protein determinations were carried out using the biuret test as proposed by Weichselbaum (58). Color was developed by adding the biuret reagent to a physiological saline solution of the extract containing 0.004 to 0.14 g. protein per 100 ml. of solution containing the biuret complex. Since proteins vary with source, it is advisable to standardize the test with protein similar in nature. A standard curve was constructed using the protein of a cell-free extract of S. lactis. The amount of protein nitrogen was determined by the Kjeldahl method. The amount of protein nitrogen was read from the standard curve (Figure 2) after a blank determination had been subtracted. The color was measured in a Klett-Summerson photoelectric colorimeter, using a filter of 555 mμ wave-length.

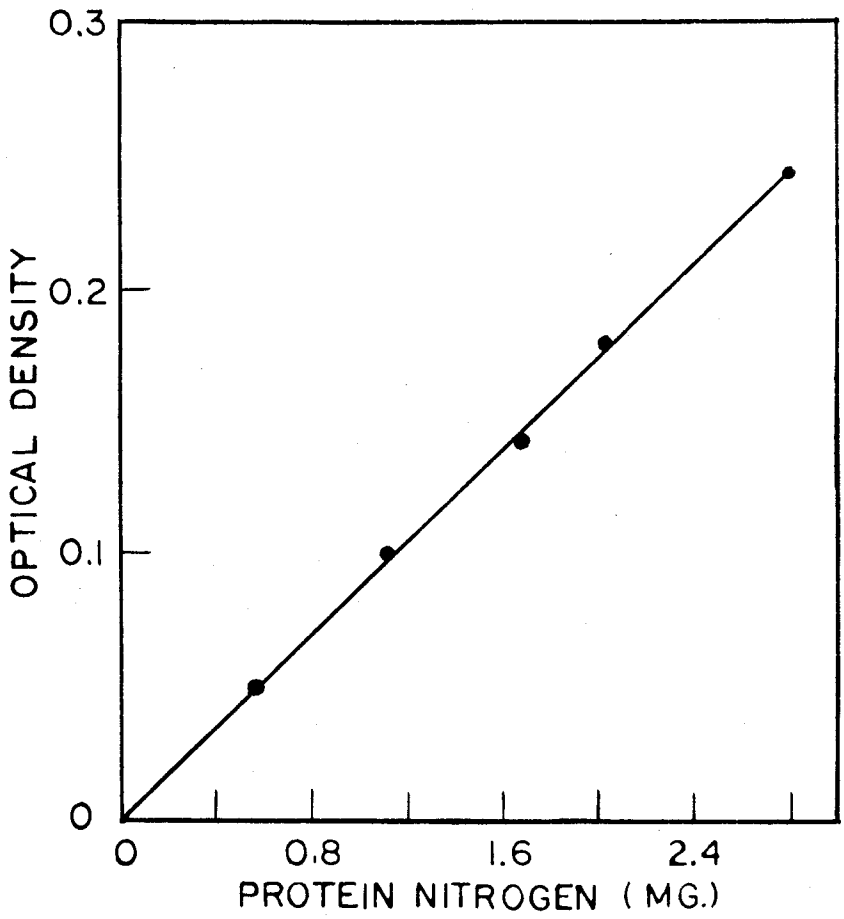

Figure 2. Standard Curve Relating Milligrams of Protein Nitrogen To Color Produced by Biuret Reagent.

Purification of commercial kaolin

Commercial kaolin was purified by several treatments with hot concentrated HCl. The iron containing solution was removed by washing with distilled water (13).

Preparation of $Al(OH)_3$ C γ

This adsorbent was prepared according to the directions given in Bertho-Grassmann's Biochemisches Praktikum (13).

Chromatography

Preparation of protein-free filtrates of milk

Protein-free fractions of skim milk after incubation with S. lactis for different lengths of time were investigated for their content of amino acids in the free- and peptide forms by paper chromatography. Protein-free filtrates of the cultures were prepared with trichloroacetic acid and processed for chromatography as described by Block (15). One l. of the sample was treated with 100 g. of trichloroacetic acid. The precipitate was allowed to form in a cold room (2° C.) for 1 hour and then was removed by centrifugation and thoroughly washed with 1 l. of cold trichloroacetic acid (2 per cent). The last traces of precipitate were removed by filtration. The combined filtrate and washings were extracted five times with ether to remove

the excess trichloroacetic acid. The aqueous layer then was passed through a 200 g. column of the cation exchange resin Duolite C-3 in the H^+ cycle at the rate of 10 ml. per minute. The resin was washed with distilled water until an aliquot of the washings was devoid of lactose, as indicated by a negative Molish test. The column then was washed with 1 l. more of distilled water as a precautionary measure. The adsorbed amino acids were eluted from the ion exchange resin with 1 l. of 4 per cent aqueous NH_3, followed by 1 l. of distilled water. This elutriate was concentrated in vacuo to 200 ml., slightly alkalinized with $Ba(OH)_2$ and again concentrated in vacuo to remove all the bound ammonia. The ammonia-free solution (negative Nessler's test) was slightly acidified with dilute H_2SO_4, the precipitate of $BaSO_4$ was removed and washed, and the filtrate and washings were evaporated to dryness. The residue was taken up in 10 ml. of 10 per cent isopropanol. An aliquot of the solution containing amino acids and peptides was hydrolyzed for 24 hours by adding 20 ml. 6 N HCl per ml. solution. The excess acid was removed by concentration in vacuo and the residue was diluted with a 10 per cent solution of isopropanol to give the same concentration of nitrogen as in the unhydrolyzed solution.

Paper chromatography procedure

Two dimensional paper chromatograms using Whatman no. 1

filter paper (7.5 in. by 7.5 in.) were carried out. The amino acid solution (0.01 ml.) was applied on the paper with a 0.01 ml. pipet on a spot in one of the corners 1 in. from the edges of the paper. The paper then was thoroughly dried with circulating air supply. Following drying, the paper was rolled into a cylinder and stapled so that the edges would not touch. Phenol (80 parts phenol and 20 parts water) and a mixture of butyl alcohol, butyric acid and water (BABW 2:2:1) were used as developing media (5). Phenol was the developing medium in the first dimension and BABW in the second dimension; development in the second dimension was repeated once. Each chromatogram was immersed 2 cm. into the developing medium, which was allowed to reach the top of the paper. Development was carried out in wide-mouth reagent bottles at room temperature. After the run in the first dimension the paper was dried and re-stapled to prepare it for the run in the second dimension. All chromatograms were run in duplicate; one was used for color development with ninhydrin, the other was viewed under ultraviolet light after heating at 100° C. for 15 minutes. Color development was carried out by spraying the paper with a solution of 1 per cent acetic acid (v/v) in 0.1 per cent ninhydrin in butyl alcohol. The paper then was dried in a hood and heated at 85° C. for 10 minutes. Known amino acids were run under the same conditions.

Colorimetric estimation of the differences in the

amount of an amino acid present in different chromatograms was performed as follows (15,28). The spots on the paper viewed under ultraviolet light were cut out and put in test tubes, followed by the addition of 1.5 ml. of ninhydrin reagent and were shaken for 5 to 10 minutes. The tubes then were placed in boiling water for 25 minutes, followed by cooling of the contents by placing the tubes in cold water. Distilled water (2.5 ml.) then was added, the tubes were shaken and acetone was added to make the volume to 10 ml. The color was measured in a Klett-Summerson photoelectric colorimeter, using a filter of 570 mμ wave-length. Differences in color extracted from spots of identical amino acids then were used to compare semi-quantitatively the amounts present.

EXPERIMENTAL RESULTS

Proteolysis by S. lactis Grown in Skim Milk

Selection of suitable strains

Thirteen pure cultures of S. lactis were isolated from different samples of raw cream. All strains were inoculated in heated skim milk and incubated at 32° C. for 24 hours. One-tenth of 1 per cent of a 24-hour milk culture was used as inoculum in all cases. Titratable acidity, plate count, nitrogen soluble and insoluble in trichloroacetic acid and total nitrogen determinations were run on each of the inoculated samples and also on an uninoculated sample (Table 1). Variations in the production of soluble nitrogen between the different strains employed were evident. Strains 18, 26 and 34 were selected for use in subsequent experiments on the basis of their ability to produce the greatest increase in acid-soluble nitrogen when grown in milk.

Increase in soluble nitrogen by three strains of S. lactis grown in milk without controlled pH

Proteolysis by three strains of S. lactis growing at 32° C. in heated skim milk for periods up to 72 hours was studied. One-tenth of 1 per cent inoculum was used in all

Table 1.

Proteolysis by Different Strains of *S. lactis* Grown in Heated Skim Milk[a]
(average of duplicate determinations)

Culture	Titratable acidity (per cent)	Standard plate count (millions/ml.)	Nitrogen in 5 g. milk (expressed as ml. 0.05 N NaOH)				Sol. N as per cent of total N
			Sol. N	Insol. N	Sum	Total N	
1	0.85	1900	5.50	33.68	39.18	39.30	13.99
4	0.85	1600	4.98	34.26	39.24	39.16	12.72
6	0.79	2000	4.80	34.50	39.30	39.45	12.17
17	0.77	1400	5.24	34.16	39.40	39.56	13.25
18	0.85	2600	5.66	33.40	39.06	39.14	14.56
26	0.89	3400	5.82	33.70	39.52	39.36	14.79
27	0.91	2000	5.50	33.70	39.20	39.26	14.04
28	0.90	2300	5.28	33.92	39.20	39.42	13.39
32	0.87	1600	5.42	33.68	39.10	39.16	13.84
33	0.88	1700	5.02	34.34	39.36	39.50	12.71
34	0.87	2500	5.62	33.40	39.02	39.20	14.34
36	0.88	1900	5.58	33.85	39.43	39.26	14.22
44	0.75	900	4.84	34.02	38.86	39.02	12.40
Control	0.15	--	4.70	34.20	38.90	39.12	12.01

[a] Incubated for 24 hours at 32° C.

cases. In Table 2 are presented representative results showing nitrogen soluble and insoluble in trichloroacetic acid, titratable acidity and plate count. In all instances the production of soluble nitrogen was fairly rapid during the first day when the cell population reached a maximum and acid production was at the most rapid rate. Later when the number of viable cells was declining and little or no acid was produced, the rate of production of soluble nitrogen also declined; however, there was a gradual increase in soluble nitrogen throughout the 72-hour test period, resulting in a conversion of 2.8 to 3.8 per cent of acid-insoluble nitrogen into the soluble form. In all cases the production of soluble nitrogen during the first 12-hour incubation period was very slight. Variations in soluble nitrogen production were evident between the three strains of S. lactis used.

Effect of calcium carbonate on proteolysis by S. lactis grown in milk

Proteolysis by two strains of S. lactis growing in heated skim milk at 32° C. with and without calcium carbonate (10 g. per 100 ml. milk) for periods up to 36 hours was studied. Calcium carbonate was added to neutralize part of the lactic acid formed by S. lactis. One-tenth of 1 per cent inoculum was used in all cases. The cultures with

Table 2.

Proteolysis by Three Strains of S. lactis Grown in Heated Skim Milk for Different Times

(average of duplicate determinations)

Culture	Age in hours	Titratable acidity (per cent)	Standard plate count (millions/ ml.)	Nitrogen in 5 g. milk (expressed as ml. 0.05 N NaOH)				Sol. N as per cent of total N
				Sol. N	Insol. N	Sum	Total N	
18	0	0.15	5.7	4.60	35.16	39.76	39.86	11.54
	6	0.23	640	4.60	35.12	39.72	39.80	11.56
	12	0.65	4000	4.69	34.93	39.62	39.70	11.81
	24	0.83	4600	5.35	34.29	39.64	39.92	13.40
	36	0.88	3000	5.48	34.38	39.86	39.85	13.75
	48	0.91	2600	5.55	34.13	39.68	39.75	13.96
	72	0.92	18	5.60	33.99	39.59	39.70	14.11
26	0	0.15	8	4.80	34.84	39.64	39.72	12.08
	6	0.18	213	4.80	34.78	39.58	39.70	12.09
	12	0.53	1620	4.83	34.69	39.52	39.62	12.19
	24	0.86	4600	5.60	34.32	39.92	39.84	14.06
	36	0.91	2400	5.85	33.71	39.56	39.70	14.74
	48	0.92	91	5.91	33.59	39.50	39.55	14.95
	72	0.92	5.1	6.00	33.42	39.42	39.60	15.15
34	0	0.15	7	4.60	35.12	39.72	39.82	11.55
	6	0.25	1300	4.60	35.12	39.72	39.70	11.59
	12	0.65	3000	4.71	35.09	39.80	39.90	11.80
	24	0.81	3700	5.50	34.30	39.80	39.94	13.77
	36	0.86	2500	5.60	34.08	39.68	39.82	14.06
	48	0.87	1000	5.65	33.87	39.52	39.68	14.24
	72	0.88	20	5.95	33.69	39.64	39.70	14.99

calcium carbonate were shaken (120 shakes per minute) throughout the incubation period by an automatic shaking device to keep the calcium carbonate suspended through the milk; one of the cultures without calcium carbonate (strain 18) also was shaken. In Table 3 are presented representative results showing increases in soluble nitrogen caused by S. lactis grown in milk with and without calcium carbonate. Addition of calcium carbonate prevented the pH from dropping below 5.0; the pH of the cultures without calcium carbonate was 4.1. Increased proteolysis was observed in the samples with calcium carbonate. After incubation for 36 hours, the increase in soluble nitrogen in culture 26 with calcium carbonate was approximately 2.5 times greater than without calcium carbonate. Shaking of the samples without calcium carbonate did not affect proteolysis. Some differences in the number of viable cells between the samples with and without calcium carbonate were found but they appeared to be only of minor importance.

Increase in tyrosine and tryptophan by three strains of S. lactis grown in milk

Proteolysis during the early phases of growth has received little or no attention, mainly because techniques commonly used such as Kjeldahl analysis for nitrogen in various soluble fractions did not show definite changes

Table 3.

Effect of Calcium Carbonate on Proteolysis by S. lactis Grown in Heated Skim Milk
(average of duplicate determinations)

Culture	Age in hours	pH	Standard plate count (millions/ml.)	Nitrogen in 5 g. milk (expressed as ml. 0.05 N NaOH)				Sol. N as per cent of total N
				Sol. N	Insol. N	Sum	Total N	
			Shaken and $CaCO_3$ Added					
18	0	6.5	7.7	4.25	35.67	39.92	40.10	10.60
	12	5.5	3500	4.77	35.38	40.15	40.25	11.85
	24	5.35	3600	6.15	33.85	40.00	40.01	15.37
	36	5.1	2000	6.30	33.49	39.79	39.89	15.79
			Shaken Without $CaCO_3$					
	0	6.5	6.7	4.22	35.98	40.10	40.05	10.54
	12	4.8	3400	4.50	35.42	39.92	39.82	11.30
	24	4.45	2700	5.19	34.83	40.02	40.15	12.93
	36	4.1	1900	5.36	34.44	39.80	39.86	13.45
			Without Shaking and Without $CaCO_3$					
	0	6.5	6.1	4.20	35.82	40.02	40.10	10.47
	12	4.6	3300	4.48	35.64	40.12	40.15	11.16
	24	4.4	3200	5.28	34.60	39.88	40.02	13.19
	36	4.1	1300	5.38	34.44	39.82	39.90	13.48

(Continued on next page)

Table 3. (Continued)

Culture	Age in hours	pH	Standard plate count (millions/ ml.)	Nitrogen in 5 g. milk (expressed as ml. 0.05 N NaOH)				Sol. N as per cent of total N
				Sol. N	Insol. N	Sum	Total N	
			Shaken and $CaCO_3$ Added					
26	0	6.7	8.1	4.55	35.33	39.88	39.89	11.42
	12	5.3	3900	5.00	34.92	39.92	40.06	12.48
	24	5.1	3700	6.42	33.34	39.76	39.72	16.16
	36	5.0	2400	7.05	32.65	39.70	39.76	17.73
			Without Shaking and Without $CaCO_3$					
	0	6.7	8.5	4.55	35.13	39.68	39.80	11.43
	12	4.7	3700	4.78	34.94	39.72	39.84	12.00
	24	4.2	3200	5.31	34.31	39.62	39.65	13.39
	36	4.1	2100	5.48	34.14	39.62	39.72	13.80

which could be associated with protein hydrolysis. The need to detect smaller degrees of protein hydrolysis in milk has led to use of a modified test for tyrosine and tryptophan discussed under Experimental Methods. This test has proved to be extremely sensitive for determining the breakdown of milk protein.

Proteolysis by three strains of S. lactis growing in heated skim milk at 32° C. for periods up to 90 hours was studied by determining the increases in tyrosine and tryptophan in trichloroacetic acid filtrates of the milk culture at certain intervals. Representative data showing pH, plate count and increases in the free tyrosine and tryptophan content of milk caused by S. lactis growing without pH control are presented in Table 4. In all cases increases in tyrosine and tryptophan were observed after a 4-hour incubation period. Approximately 35 (strain 18), 40 (strain 26) and 57 (strain 34) per cent of all tyrosine and tryptophan liberated during the full period of examination was freed during the first 12-hour incubation period, during which bacterial population and rate of acid production reached maxima, although some acid production continued subsequently. Definite increases in tyrosine and tryptophan were found when the production of soluble nitrogen, as determined by the Kjeldahl procedure (Table 2), was still negligible. Although a gradual increase in tyrosine and tryptophan was found throughout the 90-hour incubation period, a

Table 4.

Proteolysis by *S. lactis* Grown in Heated Skim Milk for Different Times[a]

Age in hours	Strain 18			Strain 26			Strain 34		
	pH	S. P. C. (millions/ ml.)	Tyrosine (γ/5 ml. milk)	pH	S. P. C. (millions/ ml.)	Tyrosine (γ/5 ml. milk)	pH	S. P. C. (millions/ ml.)	Tyrosine (γ/5 ml. milk)
0	6.60	5.8	0	6.65	31	0	6.60	4	0
4	6.30	640	30	6.25	510	30	5.95	75	50
8	4.75	2500	65	4.80	2200	90	4.30	1100	135
12	4.35	4000	95	4.40	3000	140	4.20	1600	190
24	4.20	2600	165	4.15	1800	225	4.10	640	265
36	4.10	2000	205	4.10	1600	280	4.10	560	295
48	4.10	320	230	4.05	175	315	4.10	56	325
72	4.10	0.9	250	4.10	0.6	340	4.05	0.2	330
90	4.10	--	270	4.10	--	350	4.10	--	335

[a] 225 γ free tyrosine was present in 5 ml. milk.

progressive decline in rate of production was noticed after number of viable cells and acid production reached or approached maximum levels. By calculation from average values (15), from 2.0 to 2.7 per cent of the total tyrosine and tryptophan was liberated in 72 hours, whereas the same figures relative to soluble nitrogen were 2.8 to 3.8 per cent (Table 2).

Effect of adding lactic acid to milk on the amount of soluble nitrogen and tyrosine and tryptophan

Sterile lactic acid added to heated skim milk to give titratable acidities ranging from 0.2 to 1.0 per cent, followed by incubation at 32° C. for 24 hours, failed to show any increase in either soluble nitrogen or tyrosine and tryptophan, indicating that the protein breakdown was not due to the lactic acid produced by S. lactis.

Proteolysis by S. lactis grown in milk with controlled pH

Strain 26 of S. lactis was grown in milk controlled at various pH levels (5.5 to 8.5) and without pH control, as outlined in the section under Experimental Methods. In Figures 3 and 4 are presented representative results showing increases in soluble nitrogen and tyrosine and tryptophan of comparable samples with and without controlled pH. The milk used contained 3.36 mg. trichloroacetic acid-soluble nitrogen and 24.44 mg. trichloroacetic acid-insoluble

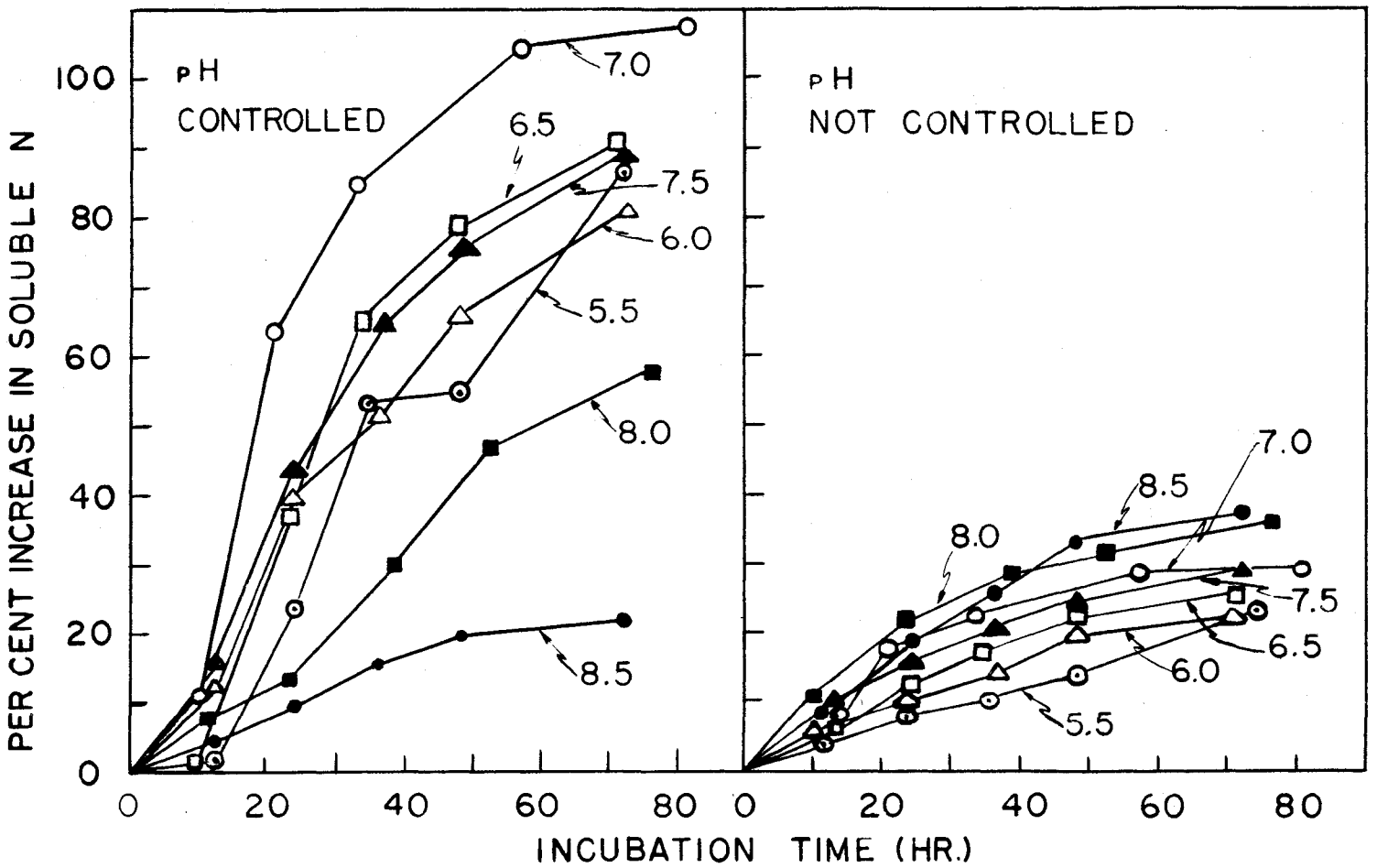

Figure 3. Increases in Soluble Nitrogen per 5 g. Sample in Milk by S. lactis 26 at Various pH Levels with and without Controlled pH (3.36 mg. Acid-Soluble Nitrogen was Present in 5 g. of the Original Milk).

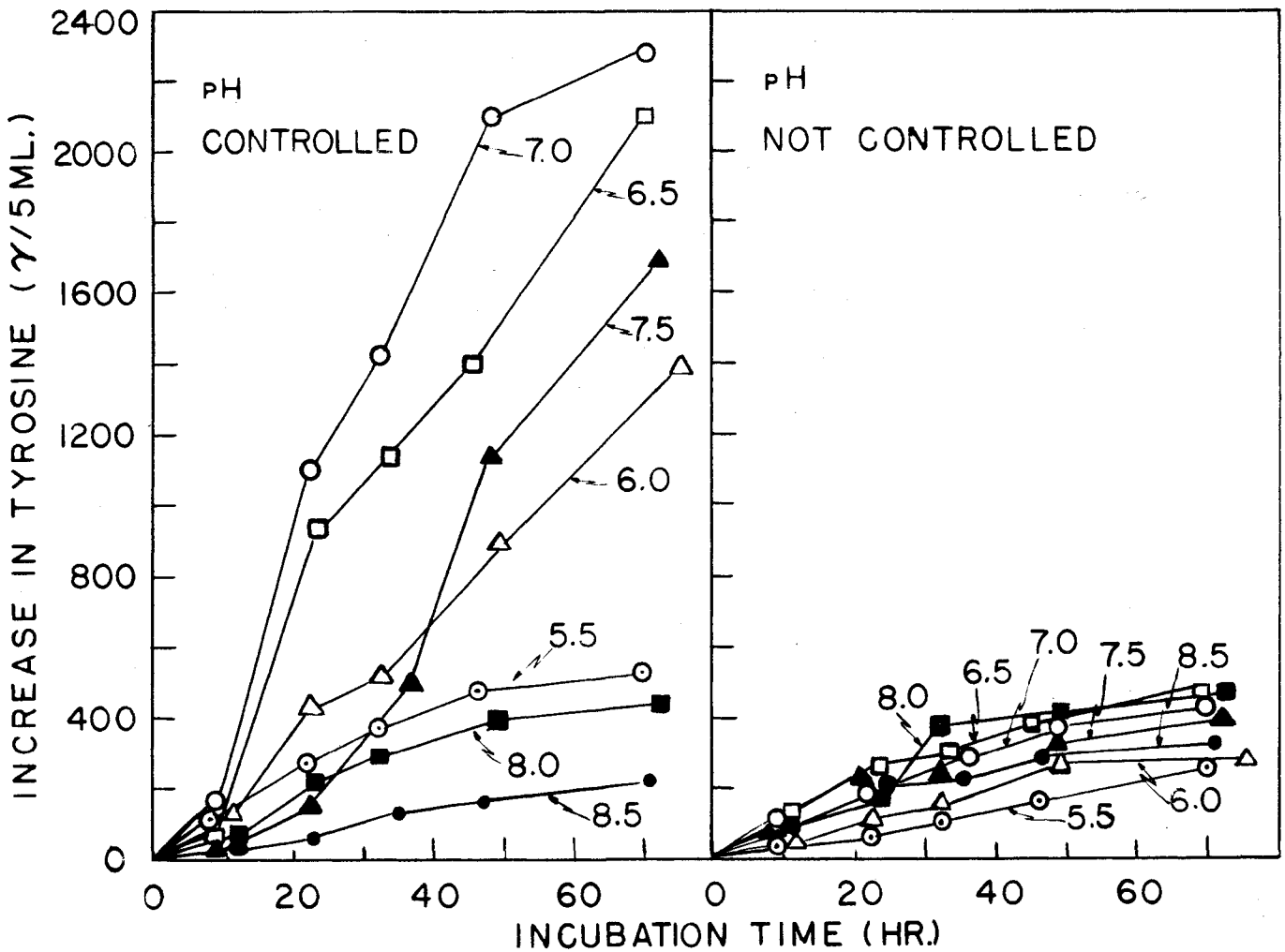

Figure 4. Increases in Tyrosine and Tryptophan per 5 ml. Sample in Milk by S. lactis 26 at Various pH Levels with and without Controlled pH (215 γ free Tyrosine was Present in 5 ml. of the Original Milk).

nitrogen per 5 g; 215 γ free tyrosine was present in 5 ml. milk.

All cultures with controlled pH showed greater increases in soluble nitrogen, except for the sample held at pH 8.5; the same was true for tyrosine and tryptophan, except for the samples held at pH's 8.0 and 8.5. The maximum increase was at pH 7.0. More soluble nitrogen and tyrosine and tryptophan were found in the samples held at pH values ranging from 6.0 to 7.5 than at pH 5.5, 8.0 and 8.5. A somewhat delayed but marked increase in soluble nitrogen was observed at pH 5.5. Although the differences between the control samples at various pH levels seemed to be only of minor importance, the amount of soluble nitrogen was greatest in the sample adjusted to pH 8.5 and decreased progressively as the medium dropped to pH 5.5, presumably because the more alkaline samples required a longer time to reach low pH levels. In the sample maintained at pH 7.0 for 70 hours (Figure 4), 18.5 per cent of the tyrosine and tryptophan present in milk proteins (15) was liberated, whereas the same figure for soluble nitrogen was 14.8 per cent (Figure 3. However the relative closeness of these figures probably is fortuitous.

In Figure 5 are presented the bacterial counts of the samples with controlled pH and their comparable controls when soluble nitrogen was determined. After the viable bacterial population had reached a maximum, little or no

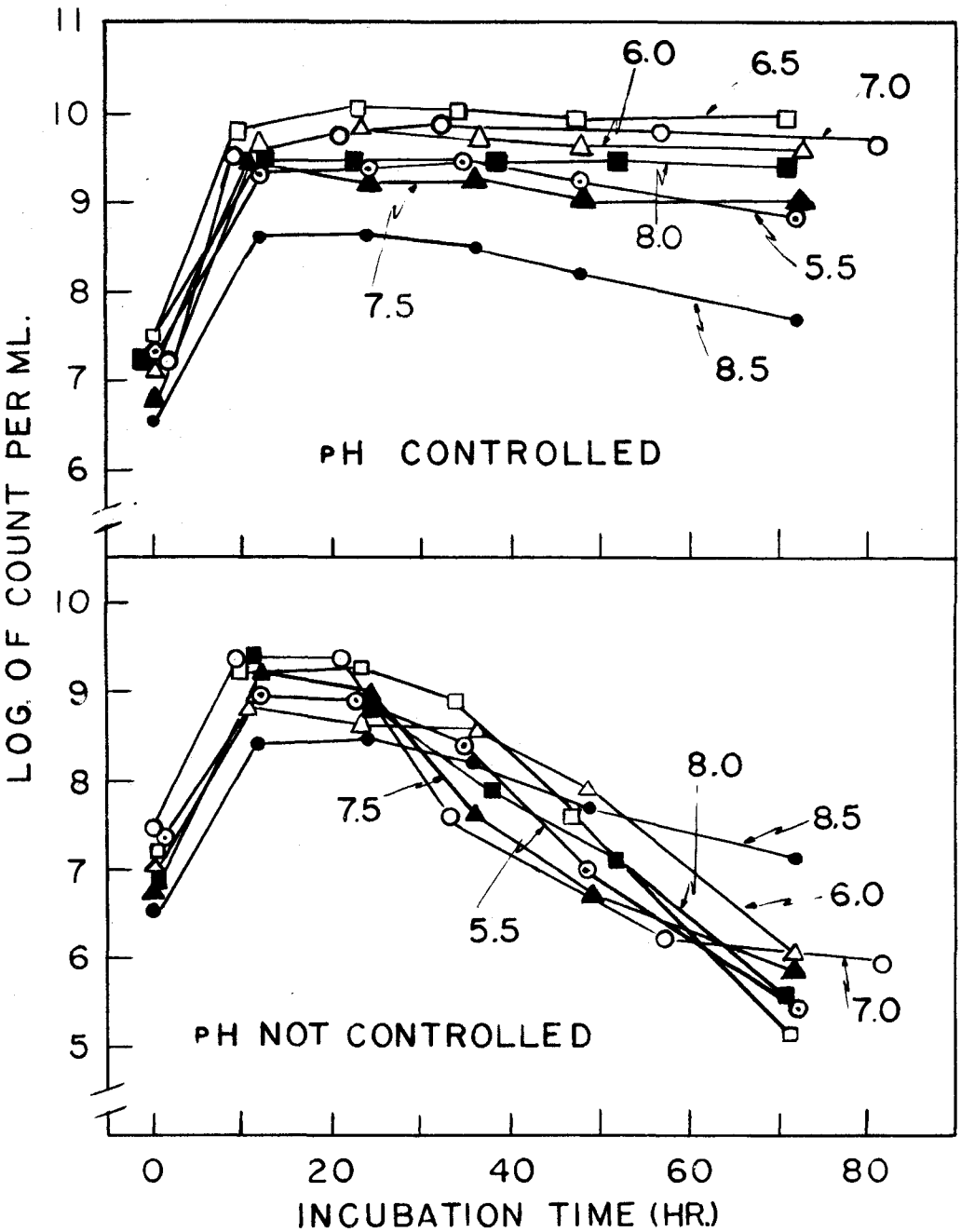

Figure 5. Population Changes of S. lactis 26 at Various pH Levels (Soluble N Expts.).

decline was observed in the samples with controlled pH; comparable controls showed a fairly rapid decline after approximately 24 hours incubation. Some differences in number of viable bacteria at different pH levels were found. The number of viable cells in the culture maintained at pH 8.5 was throughout the experimental period much lower than at the other pH values, probably because of this high pH. Some portion of the low enzyme activities may be attributed to reduced cell populations at some pH levels.

Similar results were obtained for the samples in which tyrosine and tryptophan was determined (Figure 6).

Examination of cell-free culture media for proteolytic activity

Since marked evidence of proteolysis was found in cultures of S. lactis after a short incubation period, the essentially cell-free culture medium was tested for proteolytic activity. Strains 18, 26 and 34 of S. lactis were grown in (a) skim milk, (b) simplified amino acid medium of Niven and Sherman, (c) vitamin-test casein medium B, (d) casein medium and (e) tryptone glucose beef extract broth. S. lactis was grown in these culture media for periods ranging from 4 to 72 hours at 32° C., both without controlled pH and maintained at pH 6.5, followed by removal of the cells in a high speed centrifuge. In the cultures maintained at pH 6.5 the growth medium was kept at the

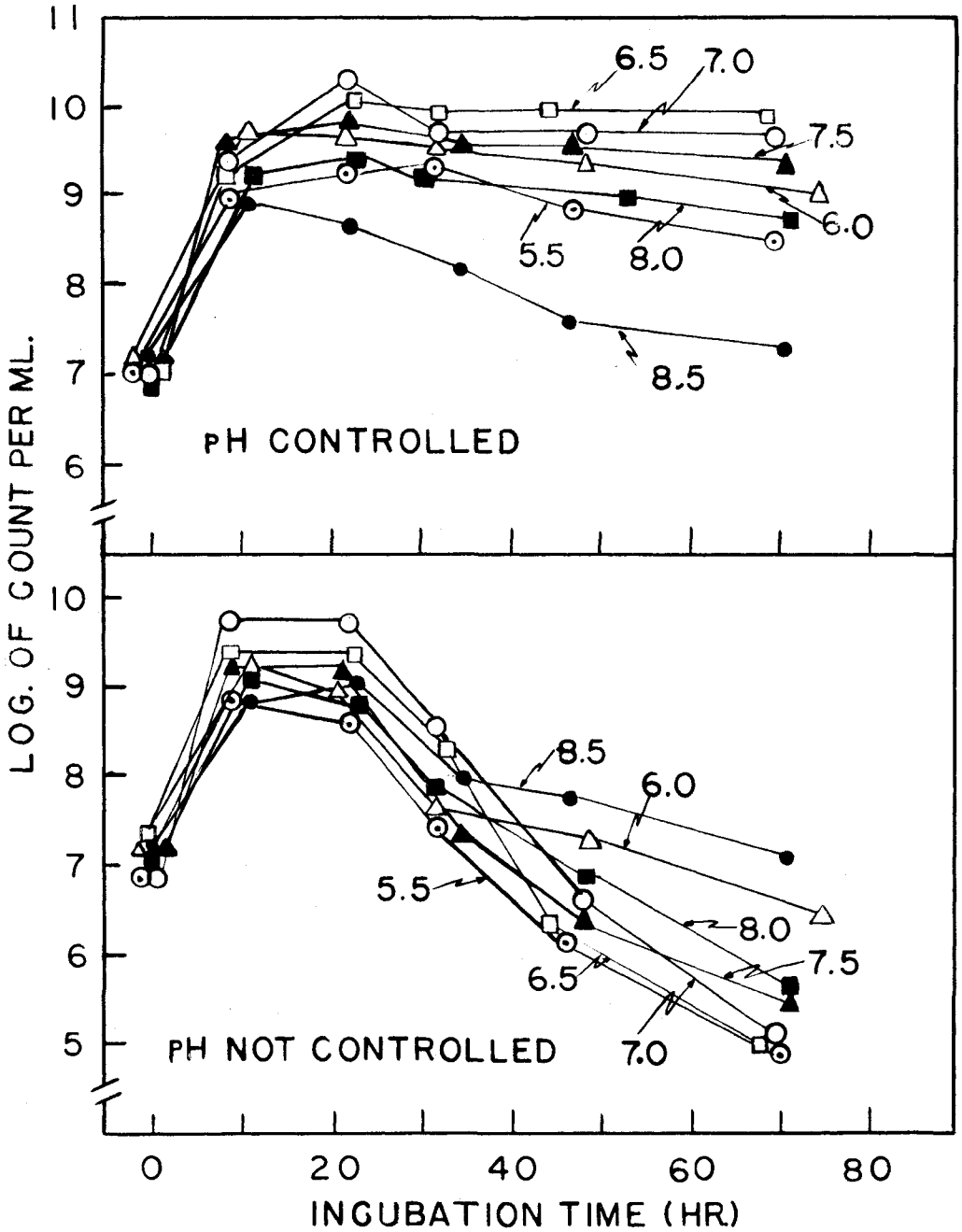

Figure 6. Population Changes of S. lactis 26 at Various pH Levels (Tyrosine Expts.).

desired pH by sodium hydroxide delivered from a Beckman automatic titrator as described previously.

Supernatant in quantities up to 5 ml. was added to 5 ml. skim milk followed by incubation at 32 and 37.5° C. for 12 to 36 hours. The skim milk was adjusted to pH values ranging from 5.0 to 8.0. Toluene, and in later experiments merthiolate (1 mg. per 40 ml. substrate) was used to prevent bacterial growth during the incubation period. Blanks were prepared by adding the supernatant to the substrate after the trichloroacetic acid has been added. No increase in soluble nitrogen or tyrosine and tryptophan was found using any of the supernatant fluid from the various cultures. If an extracellular enzyme system is produced under these experimental conditions, it obviously either is not stable or is inactive under the conditions used for determination.

Studies on the Endocellular Proteinase Activities of *S. lactis* Grown in Milk

Activity of cell-free extracts prepared by freezing and thawing, and grinding

Cell-free extracts of strain 26 of *S. lactis* grown in milk were prepared by alternate freezing and thawing and also by grinding with ground pyrex glass and alumina as described under Experimental Methods. Proteolytic activity of these extracts was determined against a casein solution

by determining the increase in tyrosine and tryptophan in 1 ml. protein-free filtrate of the digestion mixture as outlined under Experimental Methods. In order to find the pH of optimum activity, cell-free extract (1 ml.) was incubated with casein substrate adjusted to pH values ranging from 5.5 to 9.0, with incubation at 37.5° C. for 18 hours. In all cases (Figure 7A) maximum activity against casein was found at pH 7.0; a sharp decline in activity occurred on either side of the optimum pH. Greater activities were observed at acid (pH 5.5 and 6.0) than at the more alkaline pH values (pH 8.0 to 9.0).

The effect of increasing quantities of cell-free extract on the digestion of casein was studied by adding varying amounts of cell-free extract up to 1.2 ml. to casein substrate, followed with incubation at pH 7.0 and 37.5° C. for 18 hours. A direct relationship between the quantity of cell-free extract used and digestion of casein was apparent at least up to 1 ml. of cell-free extract (Figure 7B). The extract prepared by grinding the cells with alumina showed a somewhat greater activity than the one prepared by grinding with ground pyrex glass. The extract prepared by alternate freezing and thawing of cells showed the smallest activity; this may be due, at least in part, to the greater amount (approximately 30 per cent) of phosphate buffer used in the extraction process.

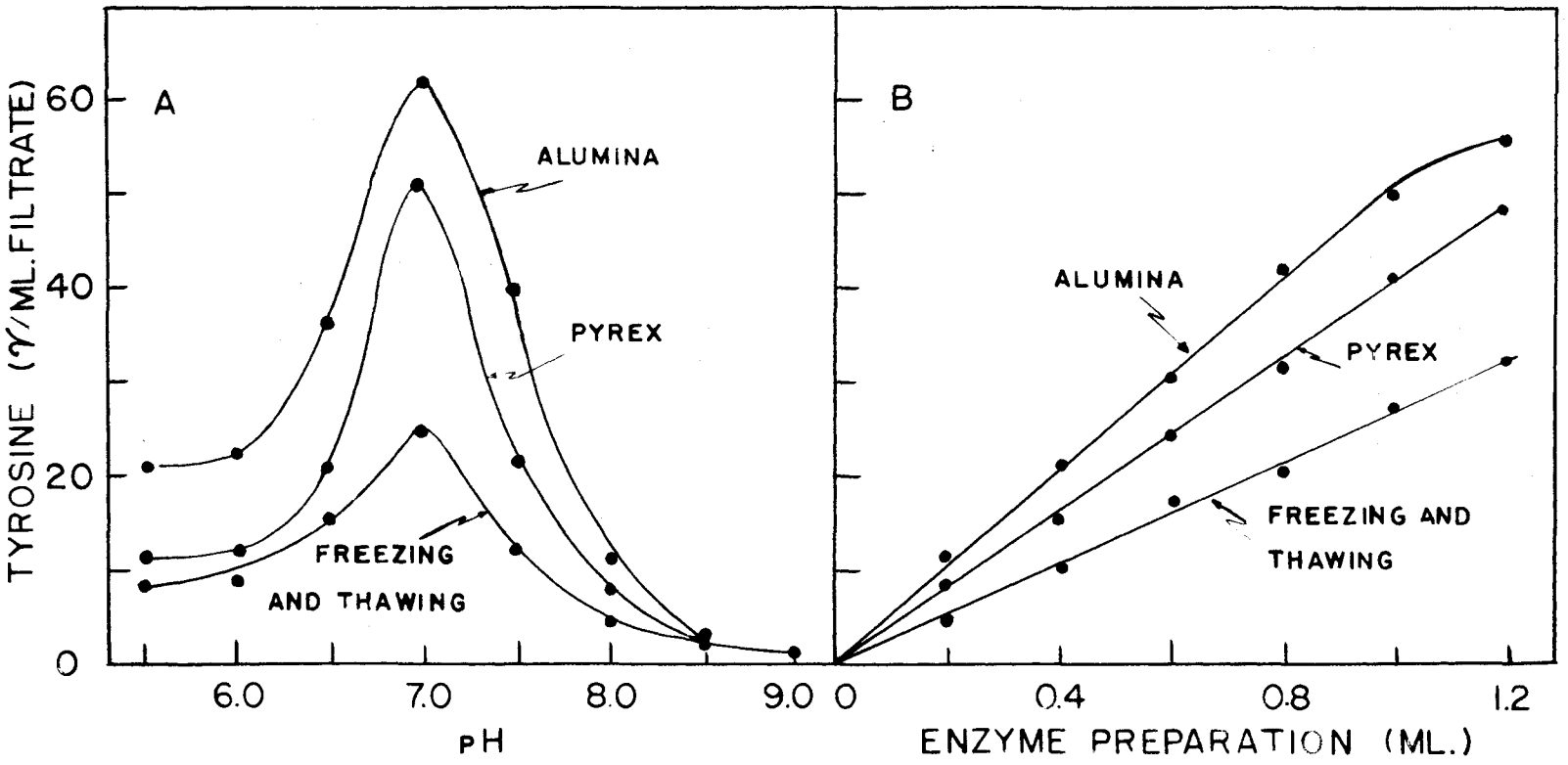

Figure 7. Proteolytic Activity (Expressed as Increase in Tyrosine) against Casein of Cell-Free Extracts Prepared in Three Different Ways from S. lactis 26, Grown in Milk. A = 1 ml. Enzyme Preparation per 5 ml. Substrate Incubated at 37.5° C. for 18 Hours; B = ml. Enzyme Preparation per 5 ml. Substrate Incubated at 37.5° C. and pH 7.0 for 18 Hours.

Preparation of cell-free extracts of S. lactis by sonic vibration

Although data in the preceding section showed that satisfactory cell-free extracts could be prepared by alternate freezing and thawing and also by grinding the cells with alumina and ground pyrex glass, more uniform extracts could be prepared by disintegration with a Raytheon Magnetostriction oscillator as described on page 25. This procedure has the additional advantage of being less complicated than the previous methods. The effect of the time of vibration on the proteolytic activity of the cell-free extract was studied. Cells of S. lactis (strain 26) were grown in heated skim milk, harvested and prepared for sonic vibration as described on pages 24-26. Identical samples were vibrated for 30, 45, 60, 75, 90 and 120 minutes. One ml. of each cell-free extract was tested for proteolytic activity against skim milk and casein (Table 5). Disintegration for a 90-minute period produced extracts with greater proteolytic activity than when shorter or longer periods were used. In all subsequent preparations vibration for a 90-minute period was used.

In the following section some of the characteristics of a cell-free extract prepared from cells of S. lactis (strains 18 and 26) grown in skim milk were studied. Cell-free extracts of S. lactis (strains 18 and 26) were prepared by

Table 5.

Effect of Time of Vibration on the Activity of the Cell-Free Extract on Skim Milk and Casein as Substrates[a]

Vibration (min.)	Tyrosine (γ per ml. filtrate)	
	Skim milk	Casein
30	39	13
45	62	26
60	82	47
75	95	52
90	105	62
120	98	59

[a] 1 ml. enzyme preparation (strain 26) per 5 ml. substrate incubated at 37.5° C. and pH 7.0 for 18 hours.

sonic vibration as discussed in the section under Experimental Methods. Proteolysis was determined by the increase in tyrosine and tryptophan in trichloroacetic acid filtrates of the substrates. Unless stated otherwise, 1 ml. of cell-free extract was added to the substrate and incubated at 37.5° C. and pH 7.0 for 18 hours.

Determination of optimum pH for proteolytic activity

In order to find the pH of optimum activity, cell-free

extract (1 ml.) was incubated with substrates which were adjusted to pH values ranging from 5.5 to 8.5. In Figures 8A and 8B are presented representative data showing the effect of pH on the digestion of skim milk, casein and lactalbumin by cell-free extracts of strains 18 and 26 of S. lactis. Maximum activity against skim milk and casein was found at pH 7.0, with some indication of a second optimum at pH 5.5. Optimum pH for lactalbumin was found at pH 6.5.

Effect of varying amounts of cell-free extract on digestion of substrates

Relationship of enzyme concentration to proteolysis of the skim milk, casein and lactalbumin substrates was studied. Different quantities of cell-free extract (up to 1.2 ml.) of both strains 18 and 26 of S. lactis were added to the substrates. Contents of each tube were made up to 7 ml. by the addition of sterile distilled water (see Experimental Methods). In Figures 9A and 9B are presented representative data showing the increases in tyrosine per ml. of protein-free filtrate by varying amounts of cell-free extract. There appeared to be a direct relationship between the quantity of cell-free extract used and digestion of skim milk, casein and lactalbumin, at least up to 1 ml. of cell-free extract.

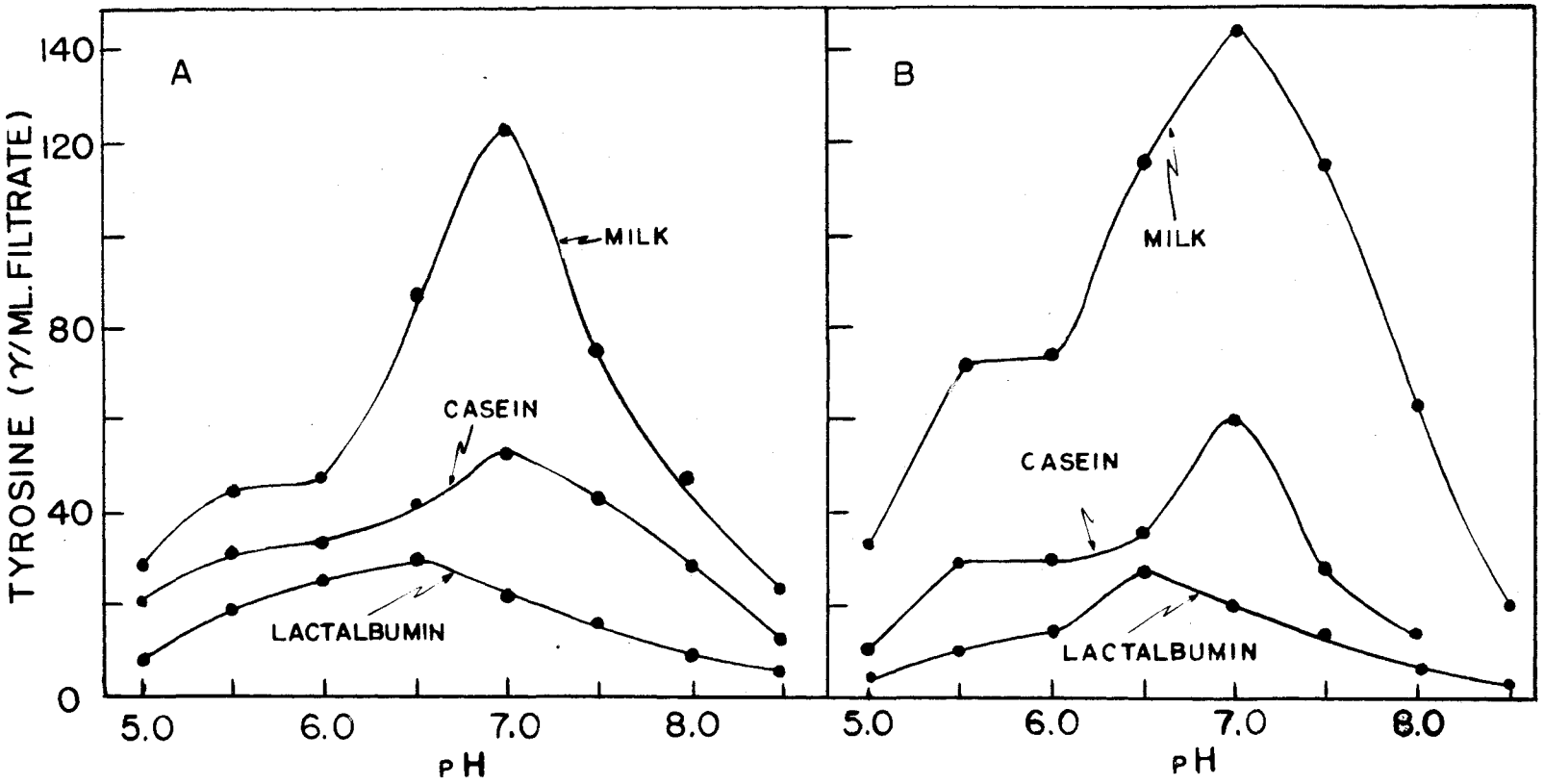

Figure 8. pH Optima for Proteolytic Activity (Expressed as Increase in Tyrosine) against Skim Milk, Casein and Lactalbumin (1 ml. Enzyme Preparation per 5 ml. Substrate Incubated at 37.5° C. for 18 Hours). A = Strain 18, Grown in Milk; B = Strain 26, Grown in Milk.

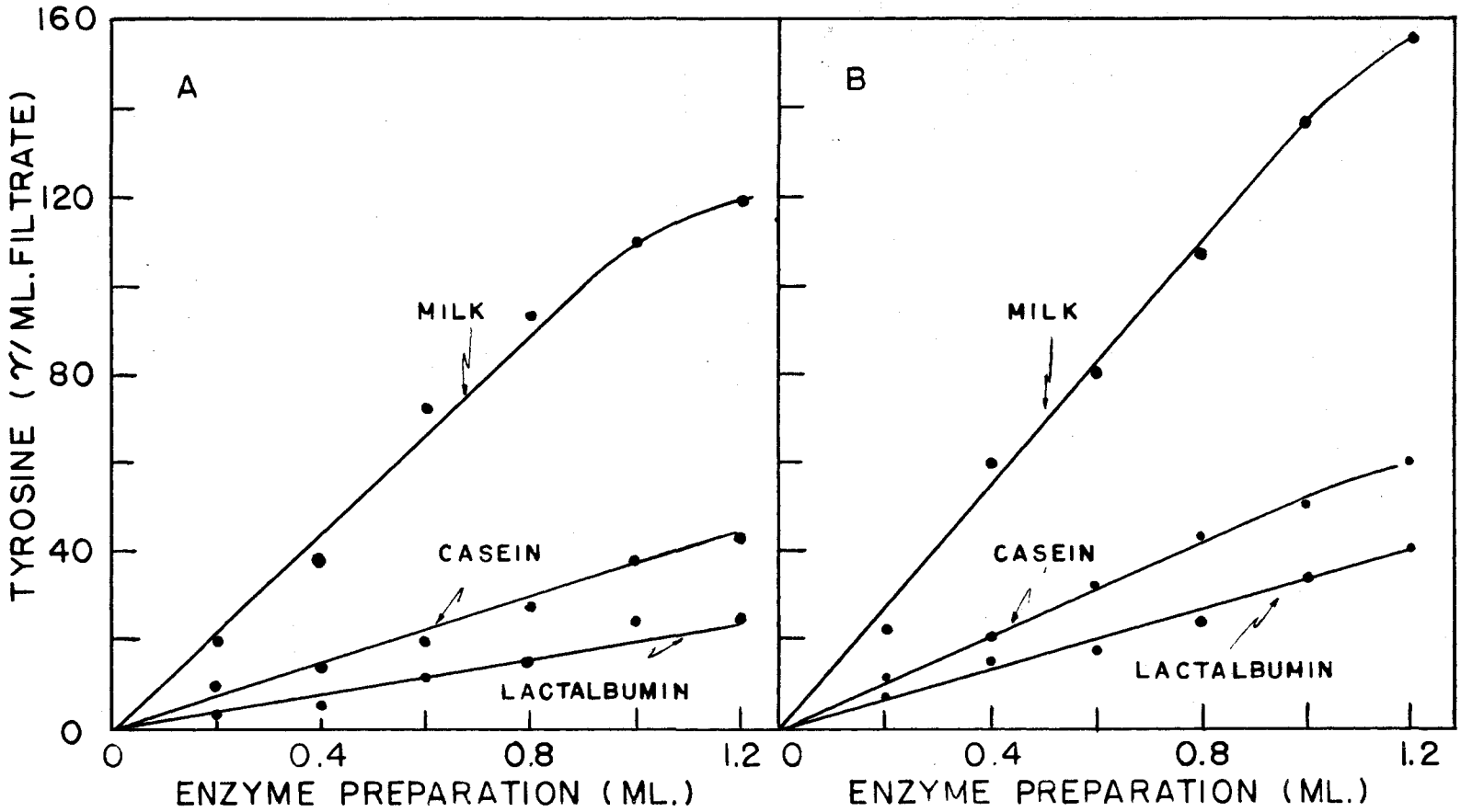

Figure 9. Effect of Increasing Quantities of Enzyme Preparation on Proteolysis (Expressed as Increase in Tyrosine) of Skim Milk, Casein and Lactalbumin (ml. Enzyme Preparation per 5 ml. Substrate Incubated at 37.5° C. and pH 7.0 for 18 Hours). A = Strain 18, Grown in Milk; B = Strain 26, Grown in Milk.

Effect of time of incubation on digestion of substrates

One ml. quantities of cell-free extract of strains 18 and 26 of S. lactis were incubated with the substrates skim milk, casein and lactalbumin for different lengths of time up to 30 hours. In Figures 10A and 10B are presented representative data showing the increases in tyrosine per ml. protein-free filtrate after incubation for different lengths of time. Up to 20 hours of incubation a direct relationship between the time of incubation and amount of digestion of all three substrates existed under the conditions of these studies.

Effect of temperature of incubation on digestion of substrates

In this case 1 ml. quantities of cell-free extract of both strains 18 and 26 of S. lactis were incubated with the substrates skim milk, casein and lactalbumin for 18 hours at temperatures ranging from 2 to 45° C. Representative data in Figures 11A and 11B show that optimum activity against skim milk, casein and lactalbumin, within the temperature limits studied, was found at 45° C.; however, the increase in values per degree of temperature increase was somewhat less above 37° C.

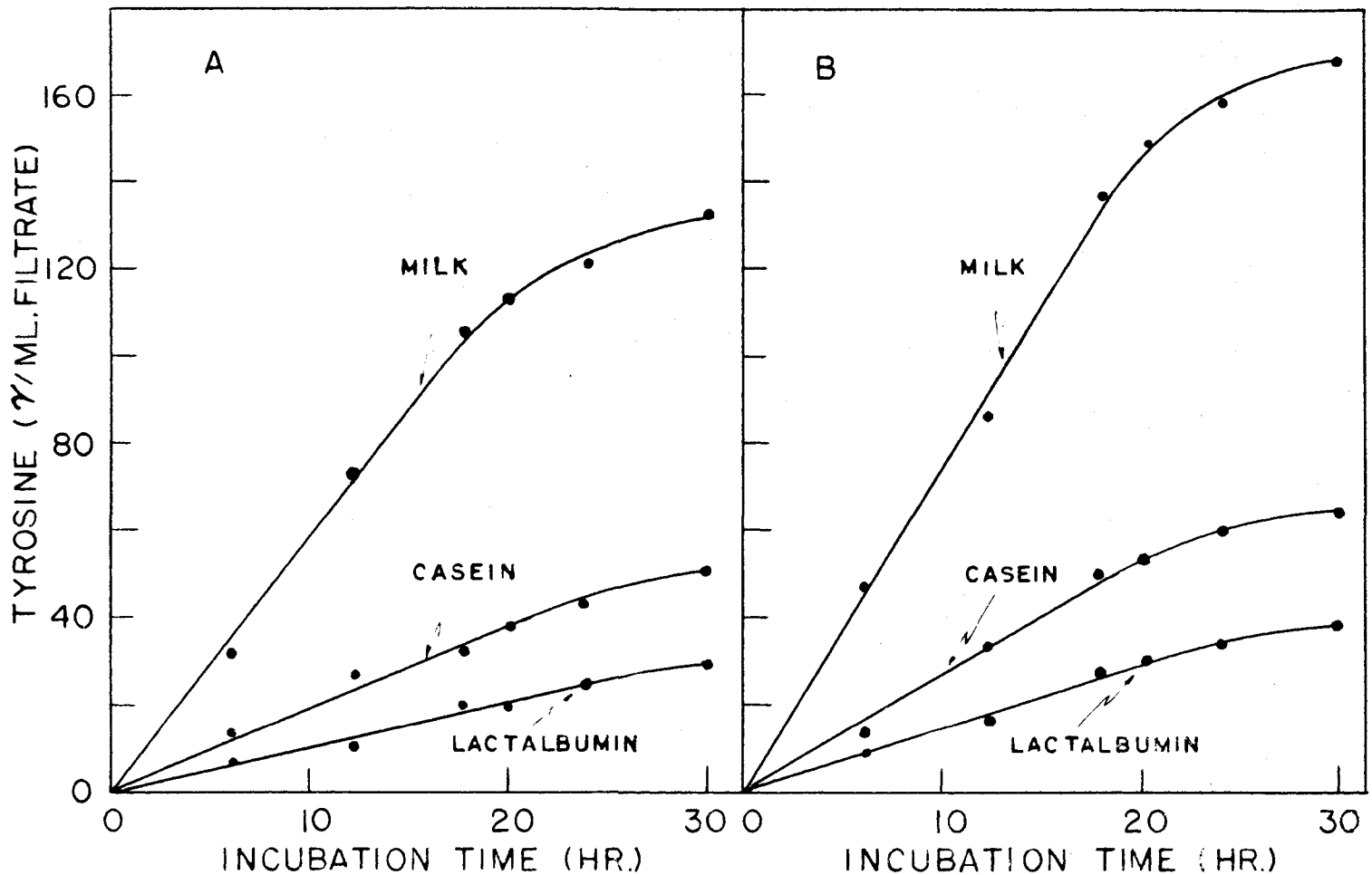

Figure 10. Effect of Time of Incubation on Proteolysis (Expressed as Increase in Tyrosine) of Skim Milk, Casein and Lactalbumin (1 ml. Enzyme Preparation per 5 ml. Substrate Incubated at 37.5° C. and pH 7.0). A = Strain 18, Grown in Milk; B = Strain 26, Grown in Milk.

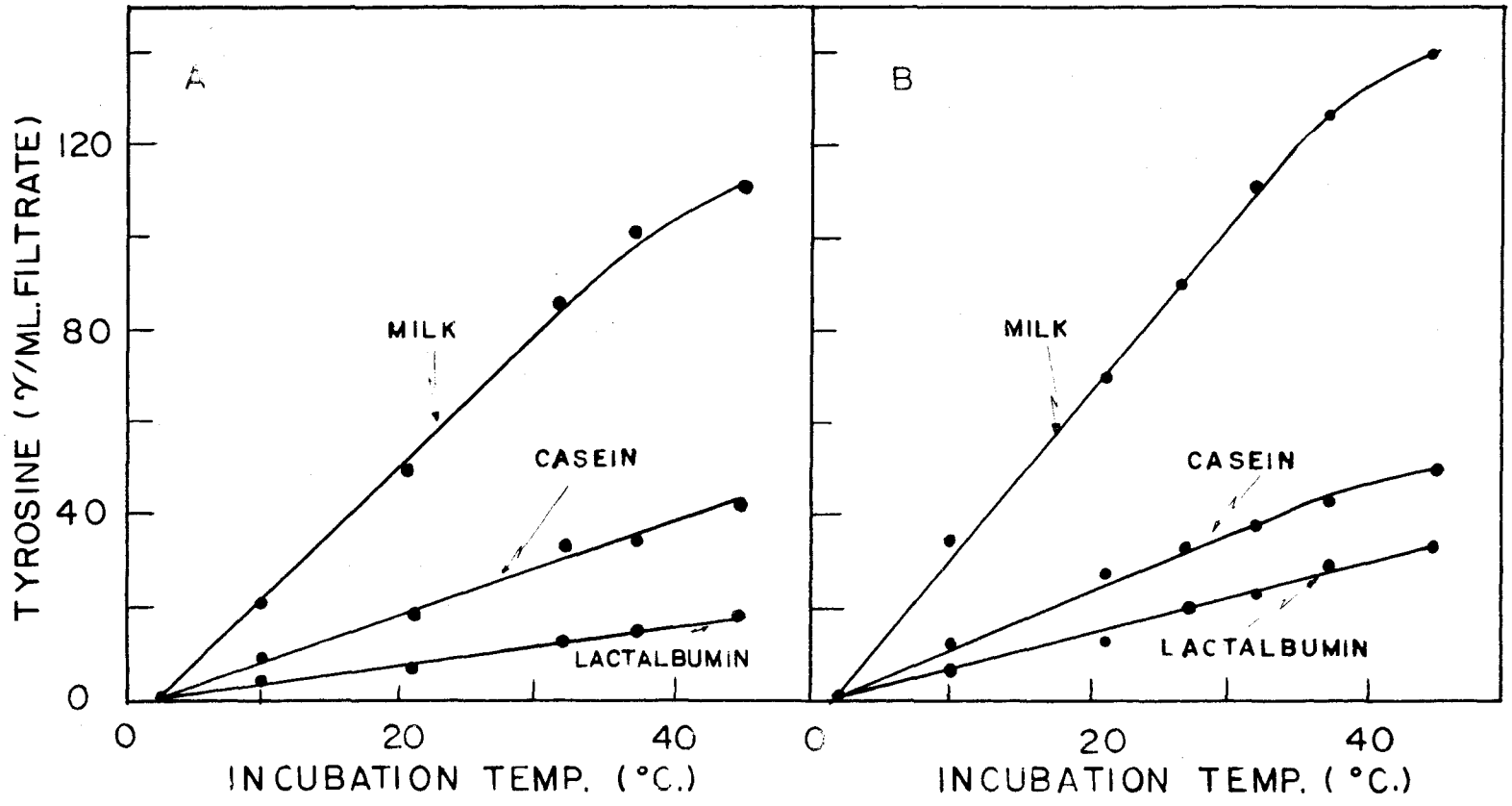

Figure 11. Effect of Temperature of Incubation on Proteolysis (Expressed as Increase in Tyrosine) of Skim Milk, Casein and Lactalbumin (1 ml. Enzyme Preparation per 5 ml. Substrate Incubated at pH 7.0 for 18 Hours). A = Strain 18, Grown in Milk; B = Strain 26, Grown in Milk.

Deriving a suitable medium for growing S. lactis cells for the preparation of cell-free extracts

As shown in the previous section, cell-free extracts of satisfactory proteolytic activity could be prepared from cells of S. lactis grown in milk. However, difficulties sometimes were encountered in separating the cells from the material collected in the bowl of the centrifuge and attempts were made to derive a broth medium for growing S. lactis cells from which extracts could be prepared with a proteolytic activity comparable to that of an extract from cells grown in milk. Strain 26 of S. lactis was grown in the following media, which were dispensed in 6 l. quantities in pyrex carboys: (a) the simplified amino acid medium of Niven and Sherman, (b) Amundstad's medium, (c) vitamin-test casein medium A, (d) vitamin-test casein medium B, (e) tryptone medium, (f) casein medium, and (g) tryptone glucose beef extract broth. All media were autoclaved for 13 minutes at 15 lb. pressure, rapidly cooled to 32° C., adjusted to pH 6.8 to 7.0, and inoculated with 1 per cent of a culture of S. lactis (strain 26), which had been transferred daily for three transfers in the culture medium employed. Incubation at 32° C. for 10 hours gave a satisfactory yield of young, metabolically active cells. During incubation the pH was kept at 6.8 to 7.0 by periodic addition of sodium hydroxide. Following incubation, the cells

were harvested and cell-free extracts were prepared as described in the section under Experimental Methods. One ml. of each cell-free extract, representing approximately 3×10^{10} cells, was tested for proteolytic activity against skim milk at pH 7.0 with incubation for 18 hours at 37.5° C. The number of viable cells per ml. medium after incubation for 10 hours and the proteolytic activity of the extracts against skim milk are presented in Table 6. The extracts prepared from cells harvested from vitamin-test casein medium B showed a proteolytic activity comparable to that of an extract prepared from S. lactis grown in milk. When

Table 6.

Proteolytic Activity of Cell-Free Extracts[a] of S. lactis 26 Grown in Different Media[b]
(skim milk test substratum)

Medium	Standard plate count (millions per ml.)	Tyrosine (γ per ml. filtrate)
Niven and Sherman	570	29
Amundstad	1100	35
Vitamin-test casein A	1200	65
Vitamin-test casein B	2000	99
Tryptone medium	1800	42
Casein medium	1400	61
T.G.E. broth	900	38

[a] 1 ml. extract represents 3×10^{10} cells.

[b] 1 ml. enzyme preparation per 5 ml. substrate incubated at 37.5° C. and pH 7.0 for 18 hours.

casein was present in the growth medium, greater proteolytic activities were found in the cell-free preparations than when peptone or tryptone were used; the smallest activity was found when proteins or peptides were omitted from the medium. All growth media containing casein supported excellent growth, as shown in Table 6. These results seem to suggest that whole protein (casein) in the growth medium stimulated the proteolytic activity of the cell-free extract. Numbers of cells growing in each of the various media also undoubtedly had an effect on final enzyme activity level.

Effect of digestion of casein in the growth medium on the activity of the cell-free extract

The results obtained in the previous section seemed to suggest that the endocellular proteinase was partially adaptive, since replacement of casein with breakdown products of protein caused definite decreases in the proteolytic activity. The possible effect of the digestion of the casein in vitamin-test casein medium A with trypsin on the proteolytic activity of the cell-free extract was investigated. Sterile 2 per cent solutions of vitamin-test casein were adjusted to pH 8.0 and digested with 0.05 per cent trypsin for periods of 10, 20 and 40 hours, followed by sterilization for 15 minutes at 15 lb. pressure. The other components of vitamin-test casein medium A then were added and the medium inoculated with S. lactis (1 per cent),

followed by incubation at 32° C. for 10 hours. Vitamin-test casein medium without tryptic digestion of casein which received the same heat treatment as the medium with digested casein was used as control. Cell-free extracts were prepared from equal volumes of cells (1 ml. of cell-free extract represented approximately 3×10^{10} cells) and tested (1 ml. quantities) for proteolytic activity against skim milk at pH 7.0 with incubation at 37.5° C. for 18 hours. The number of viable cells per ml. medium after incubation and the proteolytic activity of the cell-free extracts against skim milk are presented in Table 7.

Table 7.

Effect of Tryptic Digestion of Casein in Medium on the Proteolytic Activity of the Cell-Free Extract[a] of S. lactis 26[b]

(skim milk test substratum)

Medium	Standard plate count (millions per ml.)	Tyrosine (γ per ml. filtrate)
No digestion	1900	60
Digestion for 10 hours	1800	25
Digestion for 20 hours	1800	25
Digestion for 40 hours	1700	22

[a] 1 ml. extract represents 3×10^{10} cells.

[b] 1 ml. enzyme preparation per 5 ml. substrate incubated at 37.5° C. and pH 7.0 for 18 hours.

Approximately the same number of viable cells (1800 x 10^6 per ml.) was harvested from the media with the tryptic digested and the undigested casein. The cell-free extracts prepared from cells grown in the medium with tryptic digested casein showed less than 50 per cent of the proteolytic activity of the extract prepared from cells grown in the vitamin-test casein medium without digested casein (Table 7). These results seem to support those of the previous section which suggested that the presence of whole protein in the growth medium stimulated the proteolytic activity of the cell-free extract.

Effect of omission of individual vitamins from the growth medium on the activity of the cell-free extract

A study of the effect of omission of individual vitamins from vitamin-test casein medium A on the proteolytic activity of the cell-free extract was made by omitting one vitamin at a time and determining the proteolytic activity of the cell-free extracts prepared from equal quantities of cells collected from these media. The media were inoculated with 1 per cent of a culture of S. lactis (strain 26) which had been transferred daily for three transfers in the culture medium employed. The media were incubated for 10 hours at 32° C.; the pH was kept at 6.8 to 7.0 by periodic addition of sodium hydroxide. Proteolytic activity was determined against skim milk at pH 7.0 with incubation at

37.5° C. for 18 hours. One ml. of cell-free extract represented approximately 4 x 10^{10} cells. The number of viable cells per ml. of medium after incubation and the proteolytic activities of the cell-free extracts against skim milk are presented in Table 8. Except for nicotinic acid, the omission of one vitamin at a time did not affect appreciably the proteolytic activity of the cell-free extract. The omission of individual vitamins from vitamin-test casein medium A affected the growth greatly.

Table 8.

Effect of Omission of Vitamins from Vitamin-Test Casein Medium A on the Proteolytic Activity of the Cell-Free Extract[a] of S. lactis 26[b]

(skim milk test substratum)

Vitamin omitted	Standard plate count (millions per ml.)	Tyrosine (γ per ml. filtrate)
All	0.1	--
Riboflavin	720	79
Ca-pantothenate	110	78
Nicotinic acid	170	59
Pyridoxine	200	76
Thiamine	710	79
Biotin	540	73
Folic acid	790	77
Vitamin B_{12}	870	78
None	1260	80

[a] 1 ml. extract represents 4 x 10^{10} cells.

[b] 1 ml. enzyme preparation per 5 ml. substrate incubated at 37.5° C. and pH 7.0 for 18 hours.

In order to verify that nicotinic acid was not merely acting as an activator for the enzyme system, varying amounts of nicotinic acid up to 1 mg. were added to a digestion mixture consisting of milk (5 ml.) and cell-free extract (1 ml.) prepared from S. lactis (strain 26) grown in nicotinic acid-deficient vitamin-test casein medium A. The digestion mixture was incubated at pH 7.0 and 37.5° C. for 18 hours. No increase in proteolytic activity was found in the mixtures with added nicotinic acid. This suggests that nicotinic acid is involved in some manner in the production rather than activation of this proteolytic activity.

Studies on the Endocellular Proteinase Activities of S. lactis Grown in Vitamin-Test Casein Medium B

Cell-free extracts of strain 26 of S. lactis were prepared as usual. Proteolytic activity was determined by the increase in tyrosine and tryptophan in trichloroacetic acid filtrates of the substrates skim milk, casein and lactalbumin after incubation with cell-free extract. Unless stated otherwise, 1 ml. of cell-free extract was added to 5 ml. substrate with incubation at pH 7.0 and 37.5° C. for 18 hours.

Determination of optimum pH

Substrates adjusted to pH values ranging from 5.0 to

9.0 were incubated with cell-free extract. Proteolytic activity was determined as described previously. In Figure 12A are presented representative data showing the effect of pH on digestion of skim milk, casein and lactalbumin by a cell-free extract of strain 26 of S. lactis. Maximum activity against skim milk and casein was found at pH 7.0; optimum pH for lactalbumin was at pH 6.0 to 6.5.

Activity of cell-free extract against α, β and γ casein

Two per cent solutions of α, β and γ casein were adjusted to pH values ranging from 5.5 to 9.0. One ml. of cell-free extract of S. lactis (strain 26) was added to 5 ml. of each substrate at the various pH values, at 37.5° C. for 18 hours. A somewhat greater hydrolysis of α casein than of β and γ casein was observed at pH 6.0 to 7.0 (Figure 12B). Maximum activity against α and γ casein was found at pH 6.5, for β casein at pH 6.5 to 7.0.

Effect of varying amounts of cell-free extract on digestion of substrates

Representative data showing the effect of varying amounts of cell-free extract up to 1.4 ml. on the digestion of skim milk, casein and lactalbumin are shown in Figure 13. There appeared to be a direct relationship between the quantity of cell-free extract used and digestion of all

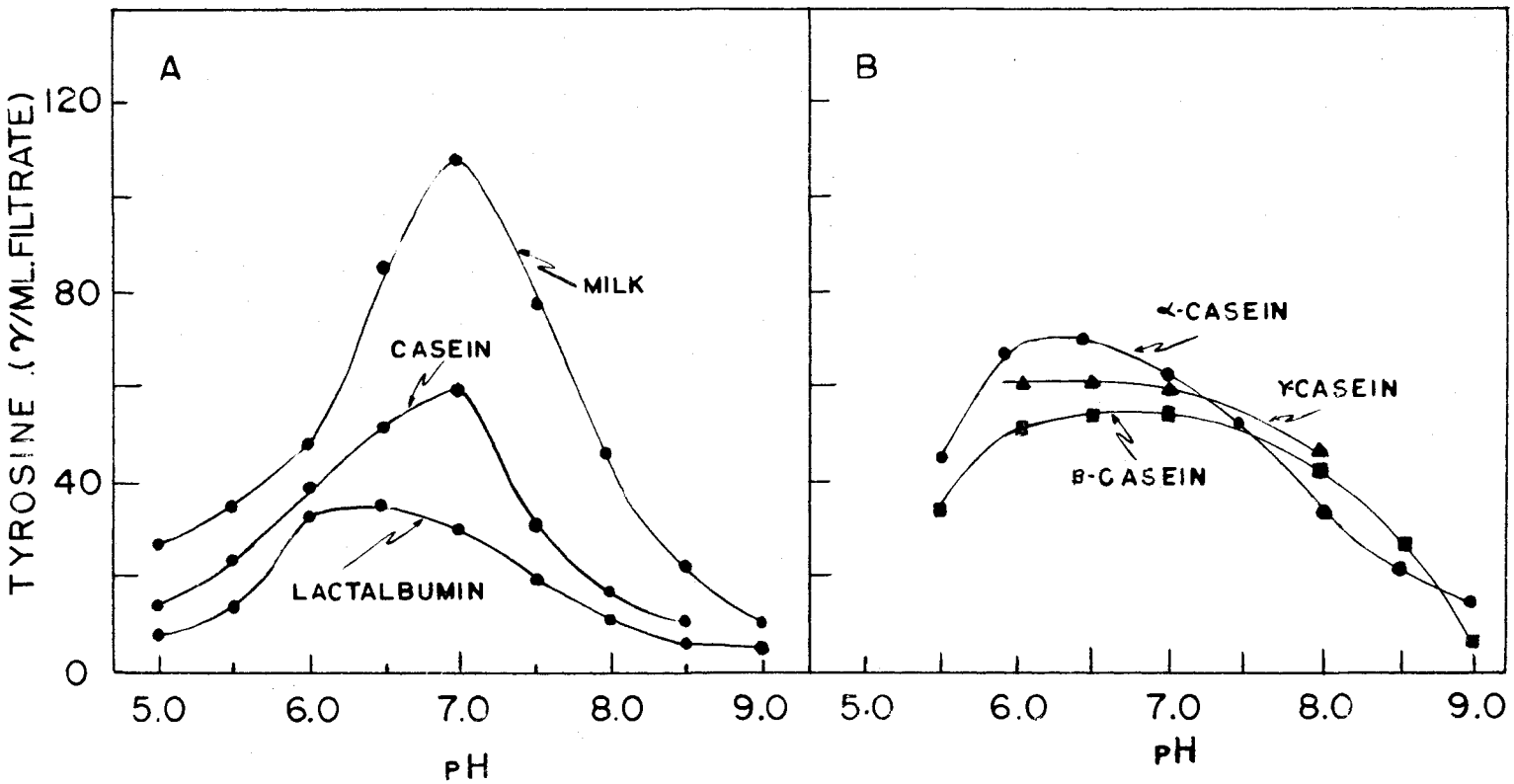

Figure 12. Effect of pH on the Proteolysis (Expressed as Increase in Tyrosine) of Skim Milk, Casein and Lactalbumin (1 ml. Enzyme Preparation of Strain 26, Grown in Vitamin-Test Casein Medium B, per 5 ml. Substrate Incubated at 37.5° C. for 18 Hours.)

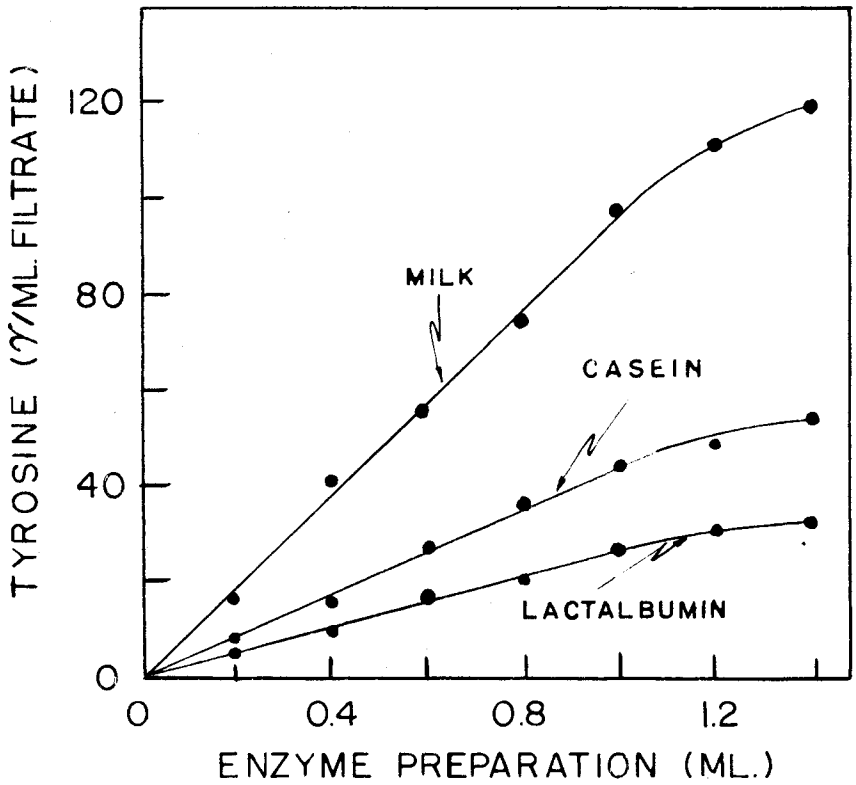

Figure 13. Effect of Increasing Quantities of Enzyme Preparation from Strain 26, Grown in Vitamin-Test Casein Medium B, on the Proteolysis (Expressed as Increase in Tyrosine) of Skim Milk, Casein and Lactalbumin (ml. Enzyme Preparation per 5 ml. Substrate Incubated at 37.5° C. and pH 7.0 for 18 Hours).

three substrates, at least up to 1 ml. of cell-free extract.

Effect of time of incubation on digestion of substrates

In this experiment 1 ml. quantities of cell-free extract were incubated with the substrates for different lengths of time up to 30 hours. In Figure 14A are presented representative data showing proteolytic activity of cell-free extract against skim milk, casein and lactalbumin when incubated for different lengths of time. Up to 20 hours of incubation, a direct relationship between the time of incubation and amount of digestion of all three substrates existed.

Effect of temperature of incubation on digestion of substrates

In this case 1 ml. quantities of cell-free extract were incubated with the substrates for 18 hours at different temperatures ranging from 2 to 45° C. Optimum activity against skim milk, casein and lactalbumin, within the temperature limits studied, was found at 45° C. (Figure 14B); however, the increase with increasing temperature was somewhat less above 37° C.

The data in Figures 12, 13 and 14, showing the effect of pH, increasing quantities of cell-free extract and time and temperature of incubation on the digestion of skim milk, casein and lactalbumin by a cell-free extract of S. lactis

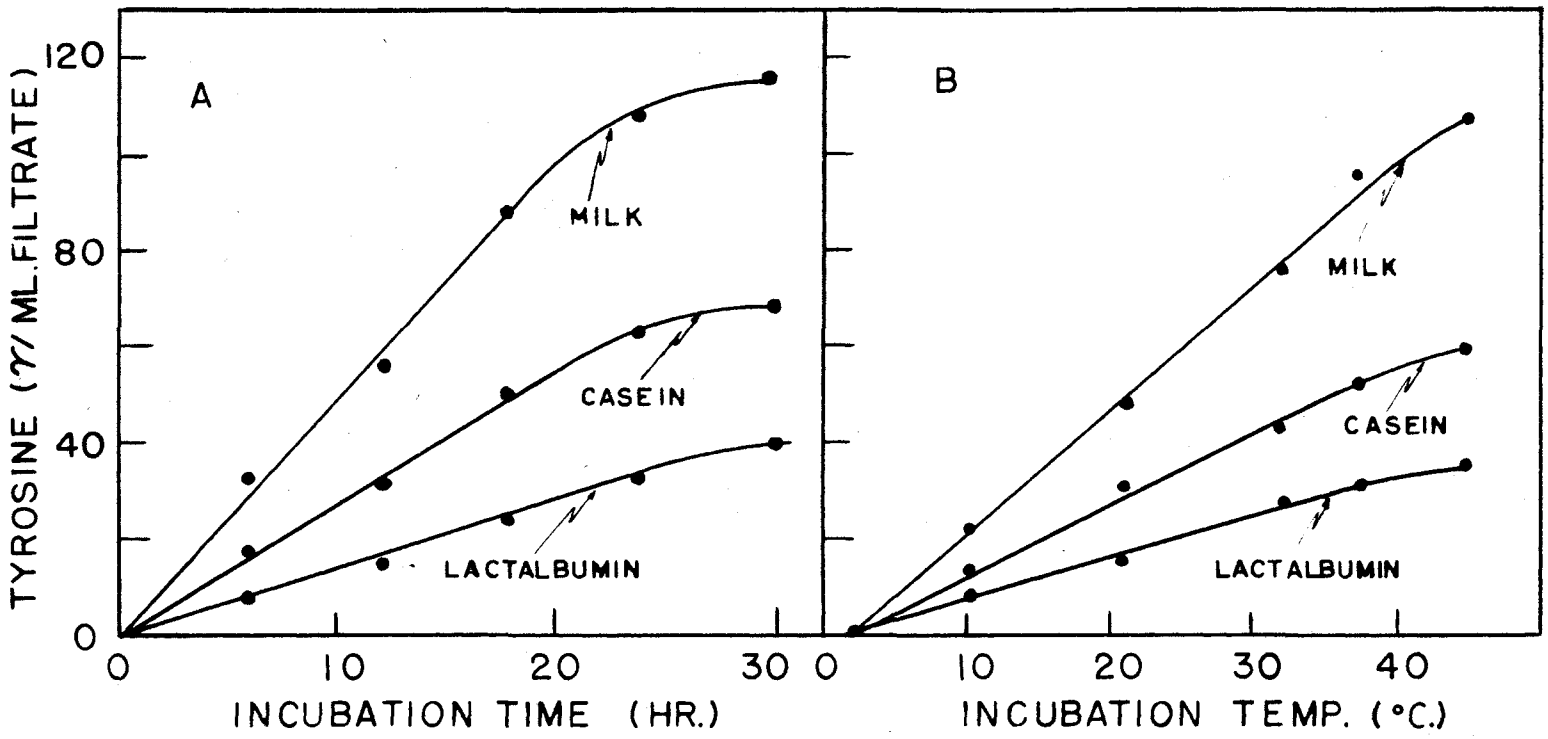

Figure 14. Effect of Time and Temperature of Incubation on the Proteolysis (Expressed as Increase in Tyrosine) of Skim Milk, Casein and Lactalbumin by Enzyme Preparation from Strain 26, Grown in Vitamin-Test Casein Medium B. A = 1 ml. Enzyme Preparation per 5 ml. Substrate Incubated at 37.5° C. and pH 7.0; B = 1 ml. Enzyme Preparation per 5 ml. Substrate Incubated at pH 7.0 for 18 Hours.

(strain 26) grown in vitamin-test casein medium B parallel closely those of cells grown in milk (Figures 8, 9, 10 and 11), except for the lack of an inflection in the curves at pH 5.5 to 6.0. The proteolytic activity of the cell-free extracts prepared from cells of S. lactis grown in vitamin-test casein medium B was somewhat less than that of an extract prepared from cells grown in milk.

Previous results (Figures 9, 10 and 11) indicate that more proteolysis was found when skim milk was used as substrate than the sum of the activities against 2 per cent solutions of casein and lactalbumin. Another study showed that the proteolysis found when cell-free extract (1 ml.) of strain 26 of S. lactis was incubated for 18 hours at 37.5° C. with a substrate (5 ml.) containing casein and lactalbumin in the proportions commonly found in milk, was smaller than when milk (5 ml.) was used as substrate. Following incubation, 1 ml. of protein-free filtrate of milk contained 132 γ of tyrosine and tryptophan, whereas the filtrate prepared from the solution containing casein and lactalbumin contained 92 γ of tyrosine and tryptophan.

Effect of reducing agents on activity of cell-free extract

Some proteolytic enzymes are known to be activated by reducing agents. The effect of some of these compounds on the digestion of skim milk by a cell-free extract of S. lactis (strain 26) was studied. One ml. quantities of

thioglycollate, Na_2SO_3, KCN and ascorbic acid in 0.1, 0.01 and 0.001 M concentrations were incubated with 1 ml. of cell-free extract at 37.5° C. and pH 7.0 for 1 hour. Following addition of substrate (5 ml. skim milk), the proteolytic activity was determined after incubation at 37.5° C. and pH 7.0 for 18 hours. The data (Table 9) show that all

Table 9.

Effect of Reducing Agents on the Proteolytic Activity of a Cell-Free Extract of S. lactis 26[a]

(skim milk test substratum)

Reducing agent	Tyrosine (γ per ml. filtrate) when reducing agent was added in concentration of:		
	0.1 M	0.01 M	0.001 M
None	96	96	98
Thioglycollate	140	118	100
Na_2SO_3	151	132	110
KCN	111	106	100
Ascorbic acid	116	102	100

[a] 1 ml. enzyme preparation per 5 ml. substrate incubated at 37.5° C. and pH 7.0 for 18 hours.

reducing agents used in 0.1 and 0.01 M concentration increased the proteolytic activity of the extract, whereas a 0.001 M concentration, except for Na_2SO_3, did not show any appreciable activation. Na_2SO_3 proved to be the best activator. No reducing agents were used in subsequent experiments.

Effect of metallic ions on activity of cell-free extract

Some proteolytic enzymes are known to be both activated and inhibited by several different metallic ions. The effect of some of these on the activity of a cell-free extract of S. lactis (strain 26) against skim milk was studied. One ml. of the following solutions in 0.1, 0.01, and 0.001 M concentrations were used: $Ca(C_3H_5O_3)_2 \cdot 5H_2O$, $Fe_2(SO_4)_3$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $CoSO_4 \cdot 7H_2O$. The cell-free extracts (1 ml.) were incubated with these solutions for 1 hour at 37.5° C. and pH 7.0, followed by incubation with skim milk at pH 7.0 and 37.5° C. for 18 hours. For the most part (Table 10) the ions had either essentially no effect (Ca^{++}) or were slightly inhibitory.

Effect of heat on activity of cell-free extract

Twenty ml. portions of a cell-free extract of S. lactis (strain 26) were adjusted to pH values ranging from 5.0 to 9.0 with dilute sodium hydroxide or sulfuric acid and carefully adjusted to the same volume (23 ml.). For each trial 3 ml. of extract were placed in a test tube and heated at 50, 55 and 61.7° C. for different lengths of time. After the heat treatment, the tube was plunged in ice water to cool the contents. One ml. portions were used to test for proteolytic activity and to prepare the blank. Proteolytic

Table 10.

Effect of Metallic Ions on the Proteolytic Activity of a Cell-Free Extract of S. lactis 26[a]

(skim milk test substratum)

Metallic ion	Tyrosine (γ per ml. filtrate) when ion was added in concentration of:		
	0.1 M	0.01 M	0.001 M
None	100	100	100
Ca^{++}	104	100	98
Fe^{+++}	87	90	92
Fe^{++}	98	98	90
Mn^{++}	90	90	90
Mg^{++}	86	92	90
Zn^{++}	85	91	92
Cu^{++}	84	90	94
Co^{++}	88	88	96

[a] 1 ml. enzyme preparation per 5 ml. substrate incubated at 37.5° C. and pH 7.0 for 18 hours.

activity was determined against skim milk with incubation at pH 7.0 and 37.5° C. for 18 hours. Maximum stability of the activity (Table 11) at all three temperatures seemed to occur at pH 7.0. Heating at 61.7° C. for only 2 minutes inactivated the preparation. The extract was inactivated quite rapidly at pH 5.0 and 9.0, even at the lower temperatures.

Table 11.

Effect of Heat at Different pH Levels on the Proteolytic Activity of a Cell-Free Extract of S. lactis 26[a]

(skim milk test substratum)

pH of extract	Tyrosine (γ per ml. filtrate[b]) after heating enzyme at:							
	50° C.			55° C.			61.7° C.	
	5 min.	15 min.	25 min.	5 min.	10 min.	15 min.	1 min.	2 min.
5	34	30	24	18	6	0	0	0
6	62	48	38	30	18	6	0	0
7	78	64	58	40	27	10	5	0
8	68	42	40	28	12	0	0	0
9	36	34	26	14	10	0	0	0

[a] 1 ml. enzyme preparation per 5 ml. substrate incubated at 37.5° C. and pH 7.0 for 18 hours.

[b] Unheated extract: 100 γ tyrosine per ml. filtrate at pH 7.0.

Stability of the proteolytic activity when held at different pH levels at 2 and 32° C.

Cell-free extracts of strain 26 of S. lactis were adjusted to pH values ranging from 5.0 to 9.0 with dilute sodium hydroxide or sulfuric acid, carefully adjusted to the same volume, and stored at 2 and 32° C. for different lengths of time up to 4 days. Following storage, the proteolytic activity of the cell-free extract (1 ml.) was determined against skim milk with incubation at pH 7.0 and 37.5° C. for 18 hours. The activity (Table 12) appeared to

Table 12.

Stability of Proteolytic Activity at Different pH Levels when Held at 2 and 32° C. for Various Lengths of Time[a]
(skim milk substratum)

Time (hours)	Tyrosine (γ per ml. filtrate[b]) after holding enzyme at pH:				
	5.0	6.0	7.0	8.0	9.0
	2° C.				
12	107	120	128	117	112
24	100	117	124	117	107
96	96	112	123	104	95
	32° C.				
12	46	102	129	98	30
24	40	83	123	102	26
96	32	80	112	83	20

[a] 1 ml. enzyme preparation per 5 ml. substrate incubated at 37.5° C. and pH 7.0 for 18 hours.

[b] Control = 128 γ tyrosine per ml. filtrate.

be quite stable between pH 5.0 and 9.0 when stored at 2° C. A considerable destruction was noted at pH 5.0 and 9.0 when stored at 32° C., and a somewhat smaller destruction at pH 6.0 and 8.0 at 32° C.

Studies on the Endocellular Peptidases of S. lactis Grown in Milk

In a previous section it was shown that the proteolytic activity of a cell-free extract of S. lactis against skim milk was destroyed at pH 7.0 by heating at 61.7° C. for only 2 minutes. Subsequent experiments showed that these heated extracts were active against various di- and tripeptides. A study of some of the characteristics of these peptidases was undertaken. Cell-free extracts of cells of S. lactis grown either in skim milk or in broth media were prepared by sonic vibration of the harvested cells as described under Experimental Methods. Unless stated otherwise, 0.2 ml. of cell-free extract was added to 3 ml. of peptide substrate and incubated at pH 8.0 and 37.5° C. for 1 hour. Hydrolysis of the peptides was determined by titrating the liberated carboxyl groups with ethanolic KOH, as described under Experimental Methods.

Influence of the pH of substrate on peptidase activity

The effect of the cell-free extract (0.2 ml.) on the

buffered solutions of five dipeptides and two tripeptides at pH values ranging from 4.0 to 10.0 was studied. In Figures 15 and 16 are presented representative data showing the effect of pH of substrate on the hydrolysis of seven peptides by cell-free extracts of strains 18 and 26 of S. lactis grown in milk. In all cases optimum activity was found between pH 7.0 and 9.0. Great differences were observed in the activity of the cell-free extract against the various peptides; the hydrolysis between pH 6.0 and 8.0 was greatest with glycyl-L-leucine, DL-alanylglycine and glycyl-L-tyrosine, and smallest with glycylglycylglycine as substrate. In most cases a rather sharp decline in activity was noticed on either side of the optimum pH; optimum activity against glycyl-L-leucine in one instance however was over a somewhat wider pH range of 6.0 to 8.0 (Figure 16). There seemed to be some tendency of the glycine peptides, when glycine carboxyl group was free, to have their optimum pH at a somewhat higher pH value than the leucine or tyrosine peptides (Figures 15 and 16). A comparison of the pH optima of the extracts from strains 18 and 26 (Figures 15 and 16) reveals that the pH for optimum activity of the extract from strain 26 against both glycyl-L-leucine and glycylglycine has shifted somewhat to the acid side and that against DL-leucylglycine somewhat to the alkaline side as compared with the optima of the extract from strain 18. These deviations were confirmed in duplicate experiments.

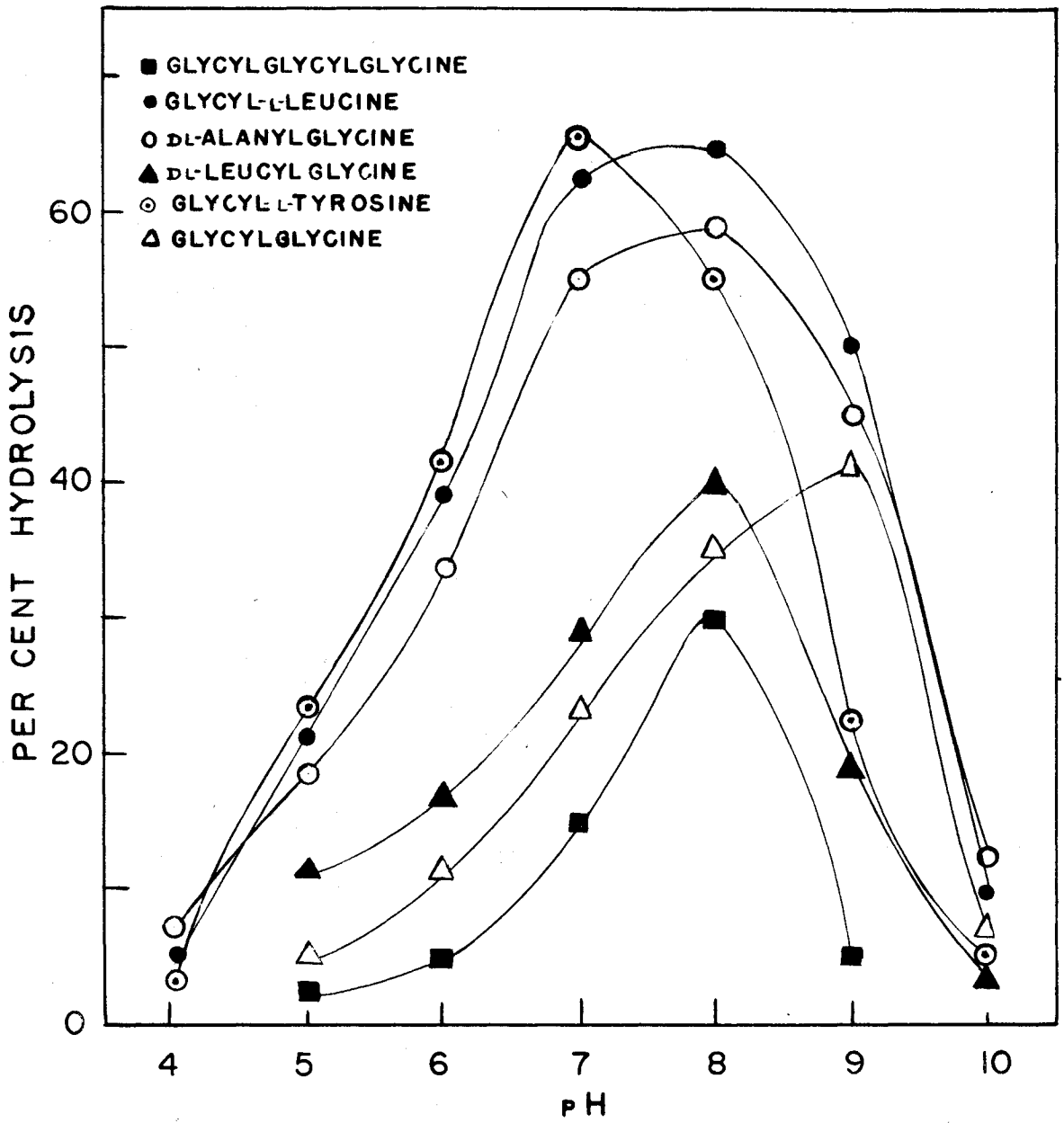

Figure 15. pH Optima for Enzyme Preparation from Strain 18, Grown in Milk, against Different Peptides (0.2 ml. Enzyme Preparation per 3 ml. Substrate Incubated at 37.5° C. for 1 Hour).

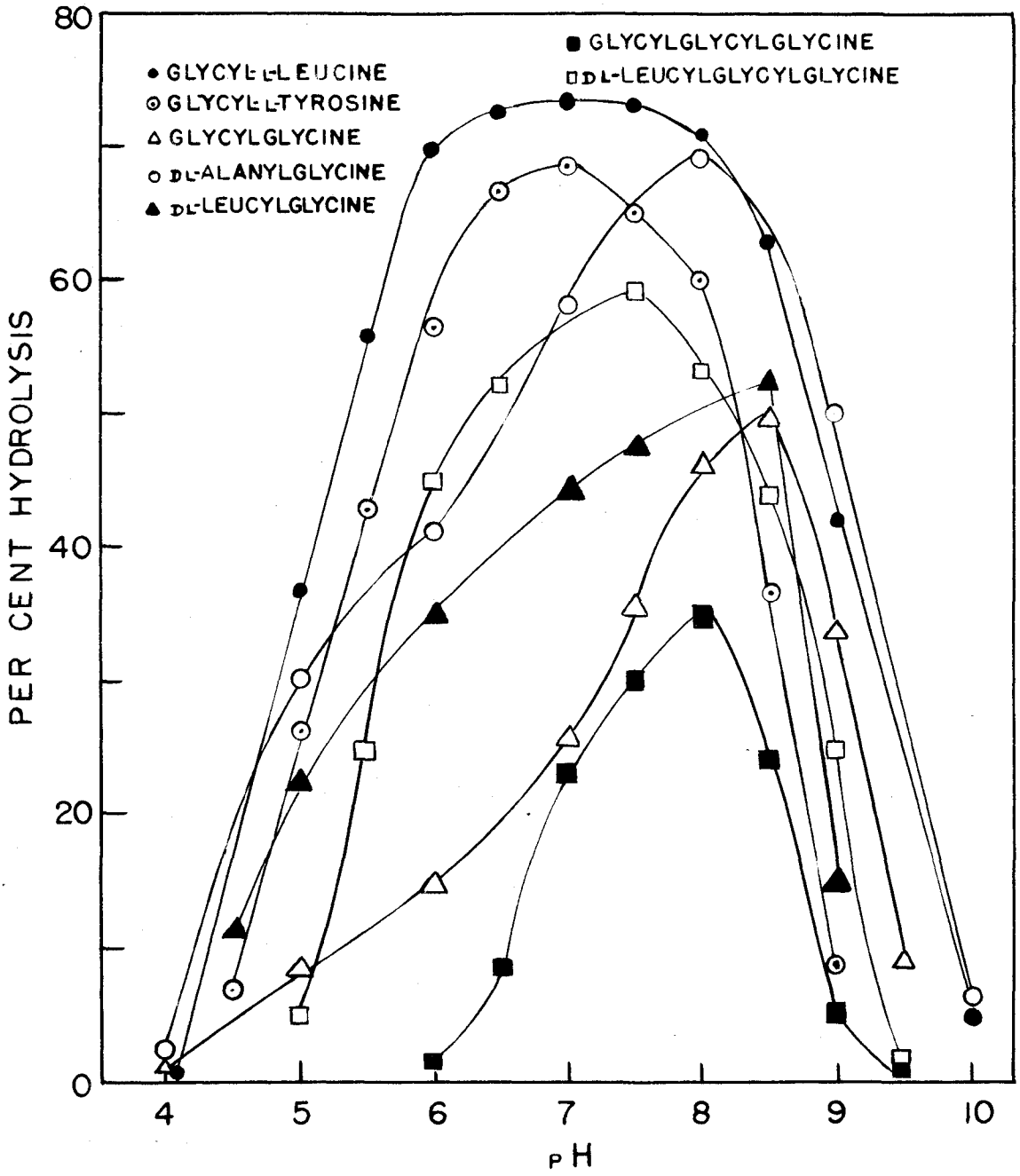

Figure 16. pH Optima for Enzyme Preparation from Strain 26, Grown in Milk, against Different Peptides (0.2 ml. Enzyme Preparation per 3 ml. Substrate Incubated at 37.5° for 1 Hour).

Effect of varying amounts of cell-free extract on hydrolysis of peptides

Increasing quantities of cell-free extract of strains 18 and 26 of S. lactis grown in milk were added to the substrates glycyl-L-leucine and DL-alanylglycine. Hydrolysis was determined at pH 8.0 and 37.5° C. after 1 hour. As shown in Figures 17A and 17B there appeared to be a direct relationship between the quantity of cell-free extract used and hydrolysis of glycyl-L-leucine and DL-alanylglycine, at least up to 0.2 ml. of cell-free extract. The activity against glycyl-L-leucine was somewhat greater than against DL-alanylglycine.

Effect of time of incubation on hydrolysis of peptides

Two-tenths ml. of cell-free extract of strains 18 and 26 of S. lactis grown in milk were incubated with glycyl-L-leucine and DL-alanylglycine as substrates for different lengths of time up to 3 hours. Up to at least 1 hour of incubation (Figures 18A and 18B) a direct relationship between the time of incubation and amount of hydrolysis of glycyl-L-leucine and DL-alanylglycine existed.

Effect of temperature of incubation on hydrolysis of peptides

Two-tenths ml. of cell-free extract of strains 18 and 26 of S. lactis grown in milk were incubated with

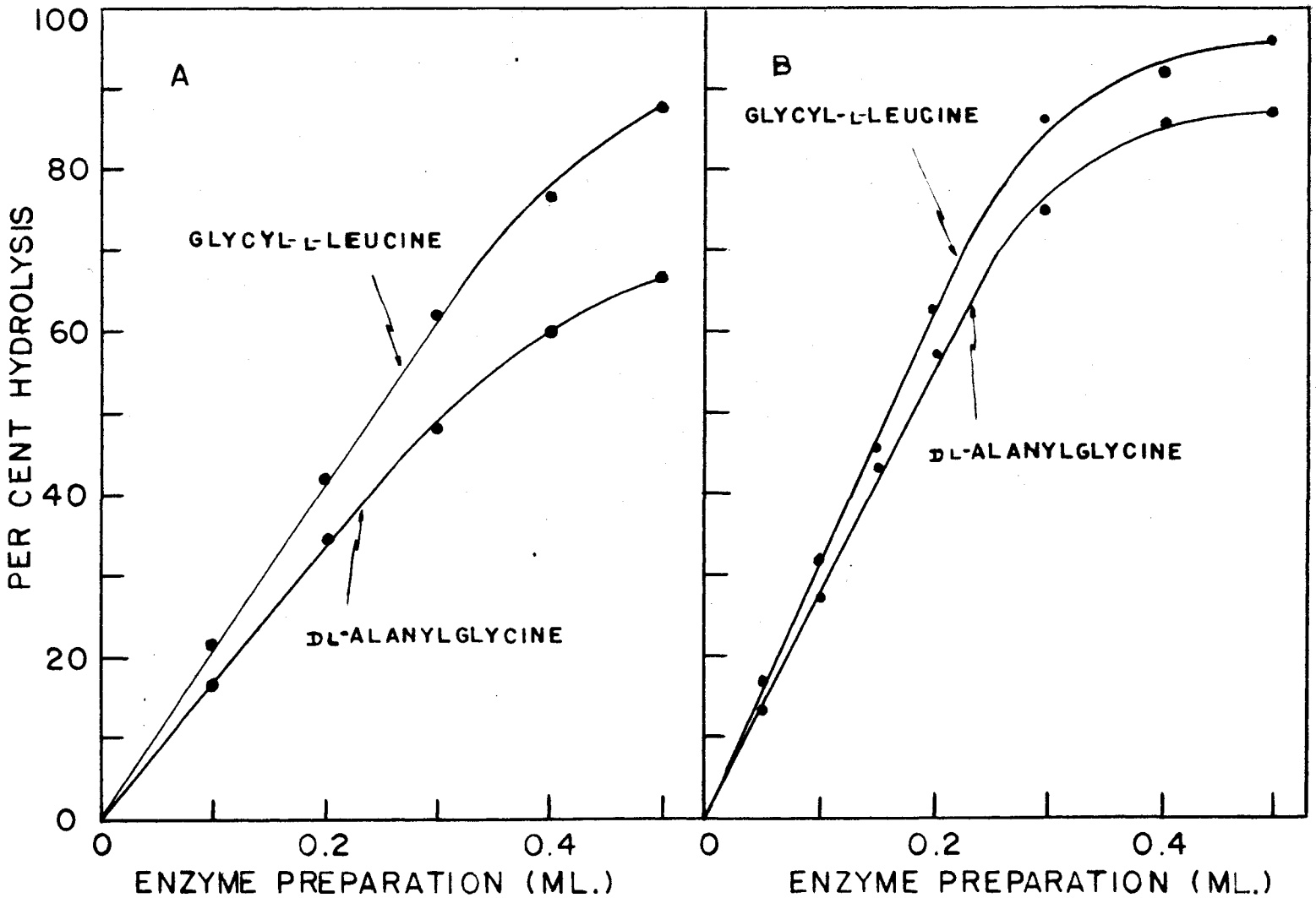

Figure 17. Hydrolysis of Peptides with Increasing Quantities of Enzyme Preparation (ml. Enzyme Preparation per 3 ml. Substrate Incubated at 37.5° C. and pH 8.0 for 1 Hour). A = Strain 18, Grown in Milk; B = Strain 26, Grown in Milk.

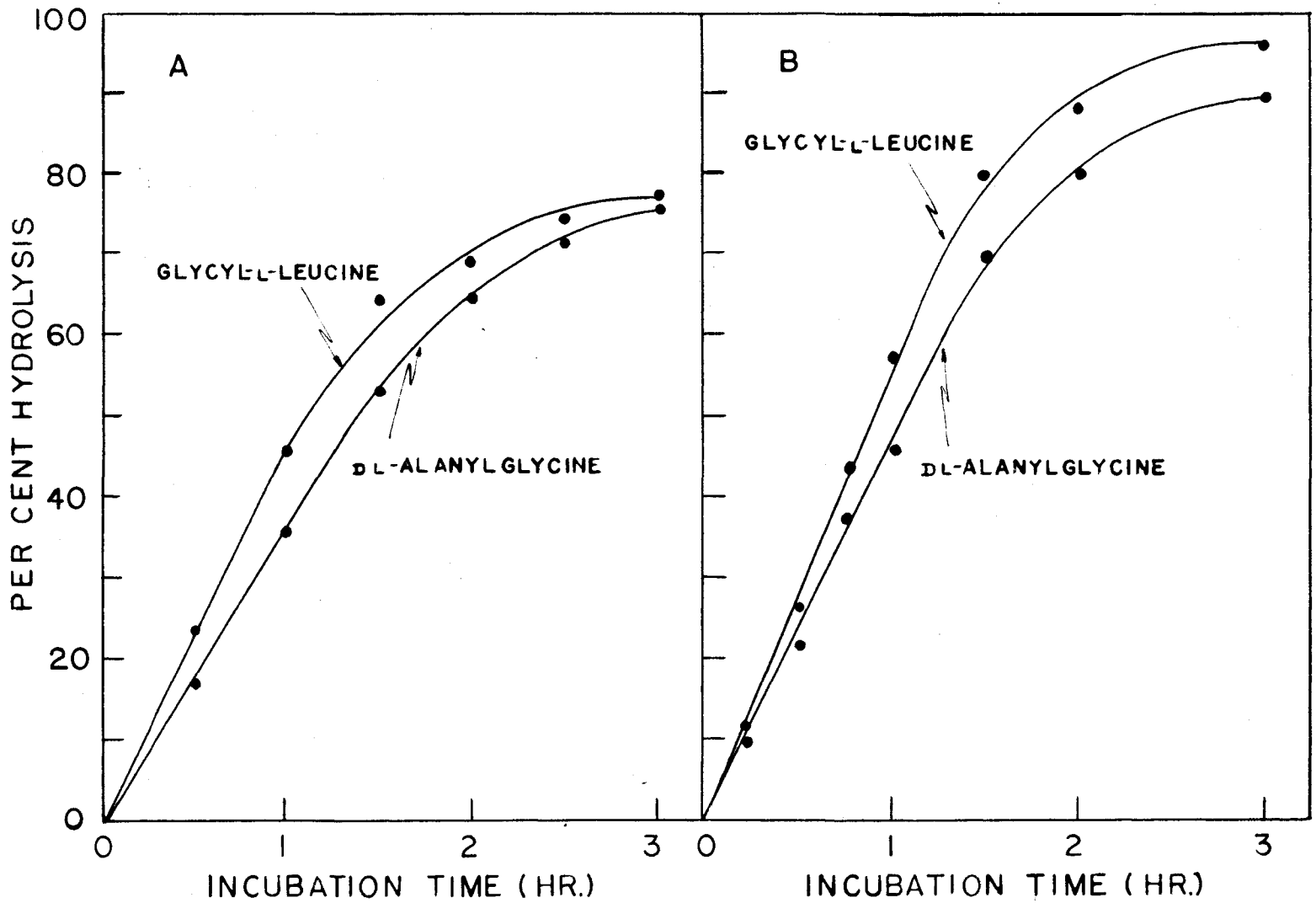

Figure 18. Effect of Time of Incubation on Hydrolysis of Peptides (0.2 ml. Enzyme Preparation per 3 ml. Substrate Incubated at 37.5° C. and pH 8.0). A = Strain 18, Grown in Milk; B = Strain 26, Grown in Milk.

glycyl-L-leucine and DL-alanylglycine as substrates at temperatures ranging from 2 to 45° C. Optimum activity against both peptides, within the temperature limits studied, was found at 45° C. (Figures 19A and 19B); however, the increase in hydrolysis with rising temperatures was somewhat less above 21° C. (strain 26) and above 30° C. (strain 18). The activity against glycyl-L-leucine was somewhat greater than against DL-alanylglycine; no activity was found at 2° C.

Study of the effect of some components of the growth medium on the production of peptidases

Cell-free extracts from strain 26 of S. lactis grown in seven different broth media employed in a previous study (page 66) were used in this experiment. Two-tenths ml. of each cell-free extract was tested for activity against glycyl-L-leucine and DL-alanylglycine with incubation at pH 8.0 and 37.5° C. for 1 hour. Representative data showing the activity of each extract against both peptides are presented in Table 13. Cell-free extracts prepared from cells of S. lactis grown in seven different media (with and without whole protein) possessed peptidase activities against glycyl-L-leucine and DL-alanylglycine comparable to those of an extract prepared from cells grown in milk.

In another experiment, the possible effect of digestion of casein in vitamin-test casein medium A with trypsin on the production of peptidases was investigated. Cell-free

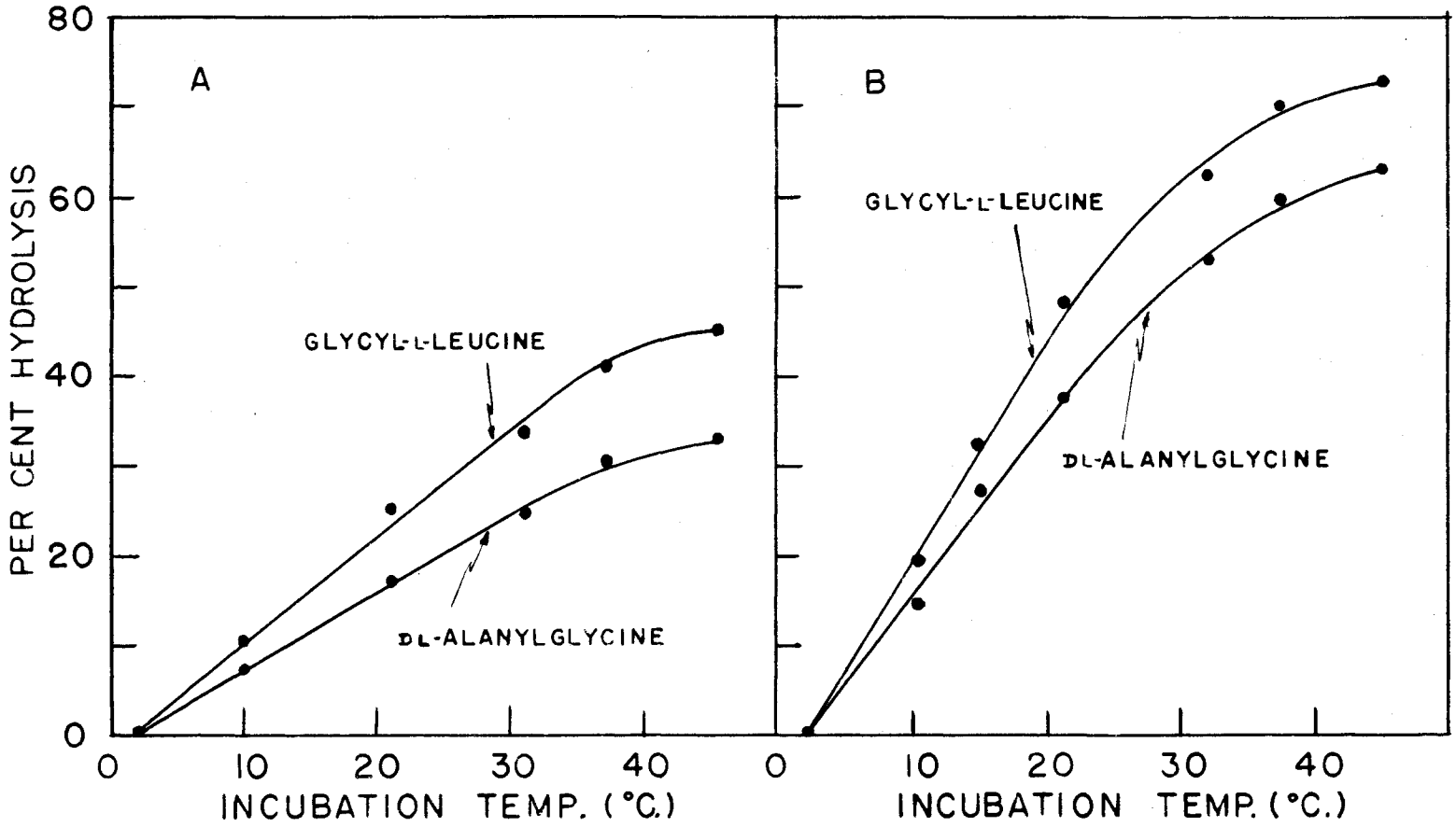

Figure 19. Effect of Temperature of Incubation on Hydrolysis of Peptides (0.2 ml. Enzyme Preparation per 3 ml. Substrate Incubated at pH 8.0 for 1 Hour). A = Strain 18, Grown in Milk; B = Strain 26, Grown in Milk.

Table 13.

Peptidase Activity of a Cell-Free Extract[a] of *S. lactis* 26 Grown in Different Media[b]

Medium	Standard plate count (millions per ml.)	Per cent hydrolysis	
		Glycyl-L-leucine	DL-alanyl-glycine
Niven and Sherman	570	56	42
Amundstad	1100	56	40
Vitamin-test casein A	1200	58	42
Vitamin-test casein B	2000	62	46
Tryptone medium	1800	58	48
Casein medium	1900	58	42
T.G.E. broth	900	56	50

[a] 1 ml. extract represents 3 x 10^{10} cells (strain 26).

[b] 0.2 ml. enzyme preparation per 3 ml. substrate incubated at 37.5° C. and pH 8.0 for 1 hour.

extracts, from strain 26 of *S. lactis* grown in vitamin-test casein medium A with and without tryptic digestion of the casein, as used in a previous experiment (pages 68 and 69), were employed in this study. Two-tenths ml. of each cell-free extract was tested against glycyl-L-leucine and DL-alanylglycine, with incubation at pH 8.0 and 37.5° C. for 1 hour. Representative data in Table 14 show that the digestion of casein in vitamin-test casein medium A did not affect the peptidase activities greatly. No indication was found that the presence of whole protein (casein) in the growth medium stimulated peptidase production or activities of the cell-free extract.

Table 14.

Effect of Digestion of Casein in Medium by Trypsin on the Activity of a Cell-Free Extract[a] Against Glycyl-L-leucine and DL-alanylglycine[b]

Medium used	Standard plate count (millions per ml.)	Per cent hydrolysis	
		Glycyl-L-leucine	DL-alanylglycine
No digestion	1900	60	56
Digestion for 10 hours	1800	52	48
Digestion for 20 hours	1800	60	48
Digestion for 40 hours	1700	56	48

[a] 1 ml. extract represents 3 x 10^{10} cells (strain 26).

[b] 0.2 ml. enzyme preparation per 3 ml. substrate incubated at 37.5° C. and pH 8.0 for 1 hour.

A study of the omission of individual vitamins from vitamin-test casein medium A on the peptidase activities of the cell-free extracts was made by omitting one vitamin at a time and determining the activity of the cell-free extracts prepared from equal quantities of cells collected from these media against glycyl-L-leucine and DL-alanylglycine. Portions (0.2 ml.) of cell-free extracts from these media used in a previous study (page 70) were used. Hydrolysis was determined at pH 8.0 and 37.5° C. after 1 hour. Representative data (Table 15) show that omission of biotin, nicotinic acid and pyridoxine decreased the activity against glycyl-L-leucine somewhat; the same was true for the omission of biotin, nicotinic acid and calcium pantothenate when

Table 15.

Effect of Omission of Vitamins from Vitamin-Test Casein Medium A on the Activity of Cell-Free Extract[a] Against Glycyl-L-leucine and DL-alanylglycine[b]

Vitamin omitted	Standard plate count (millions per ml.)	Per cent hydrolysis	
		Glycyl-L-leucine	DL-alanylglycine
All	0.1	--	--
Riboflavin	720	60	46
Ca-pantothenate	110	56	42
Nicotinic acid	170	50	40
Pyridoxine	200	52	52
Thiamine	710	70	46
Biotin	540	50	43
Folic acid	790	60	52
Vitamin B_{12}	870	62	46
None	1260	62	50

[a] 1 ml. extract represents 4×10^{10} cells (strain 26).

[b] 0.2 ml. enzyme preparation per 3 ml. substrate incubated at 37.5° C. and pH 8.0 for 1 hour.

DL-alanylglycine was used as substrate, although some of these may be borderline cases, so far as the differences being real is concerned.

Studies on the Endocellular Peptidases of S. lactis Grown in Vitamin-Test Casein Medium B

Cell-free extracts of strain 26 of S. lactis grown in vitamin-test casein medium B were prepared as described under Experimental Methods. Unless stated otherwise, 0.2 ml. of cell-free extract was added to 3 ml. of peptide substrate with incubation at pH 8.0 and 37.5° C. for 1 hour.

Influence of the pH of substrate on peptidase activity

In Figure 20A are presented representative data showing the effect of pH of substrate on the hydrolysis of six different peptides by a cell-free extract of strain 26 of S. lactis. In all cases optimum activity was found between pH 7.0 and 8.0. In most cases a rather sharp decline in activity was noticed on either side of the optimum pH; optimum activity against glycyl-L-leucine and DL-alanylglycine were over a somewhat wider pH range. Great differences were observed in the activity of the cell-free extract against the various peptides; the hydrolysis between pH 6.0 and 8.0 was greatest with glycyl-L-leucine and smallest with glycylglycylglycine as substrate. Optimum pH for DL-leucylglycine

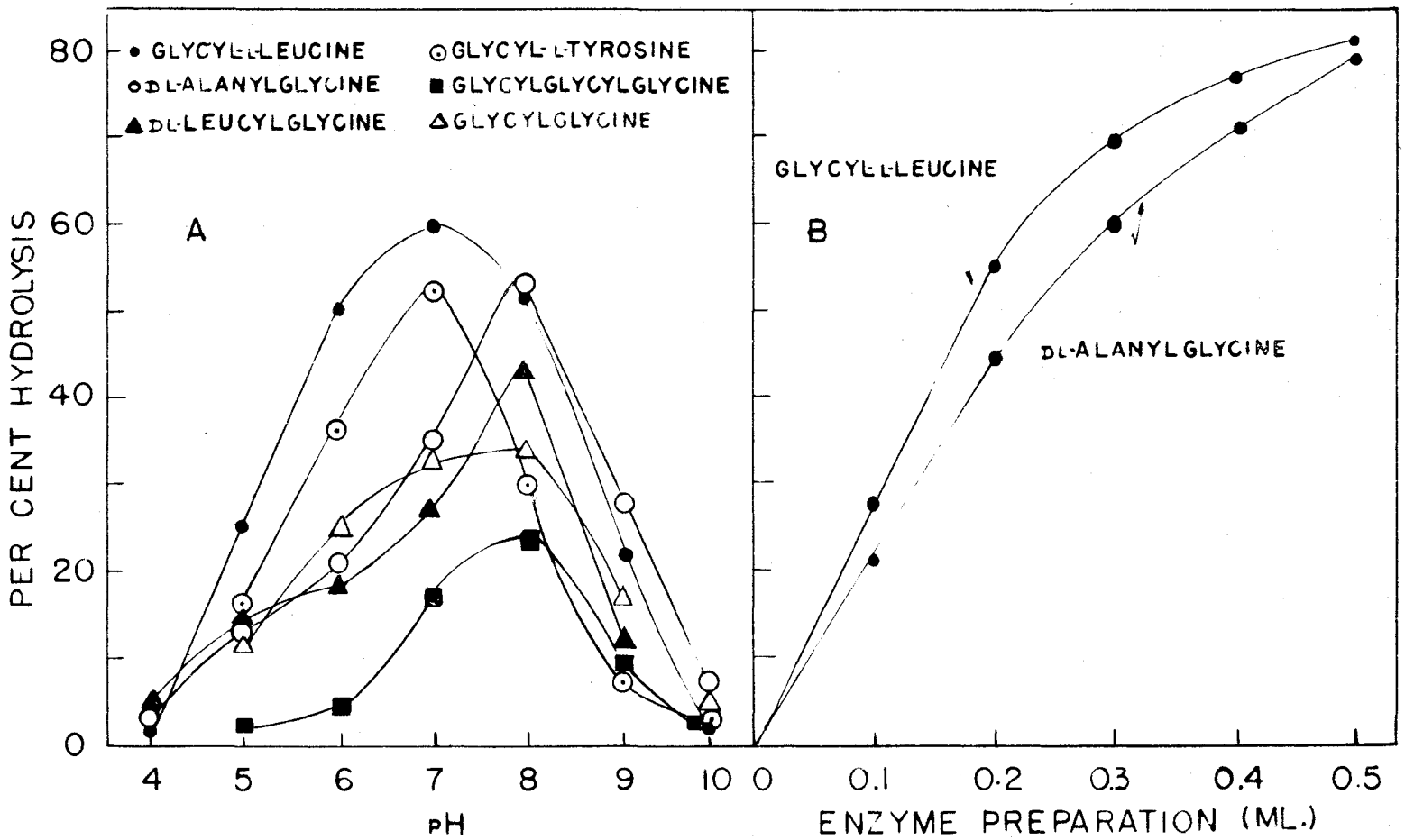

Figure 20. Effect of pH and Increasing Quantities of Enzyme Preparation from Strain 26, Grown in Vitamin-Test Casein Medium B, on Hydrolysis of Peptides. A = 0.2 ml. Enzyme Preparation per 3 ml. Substrate Incubated at 37.5° C. for 1 Hour; B = ml. Enzyme Preparation per 3 ml. Substrate Incubated at 37.5° C. and pH 8.0 for 1 Hour.

and glycylglycine was 0.5 of a unit lower than the values found for an extract prepared from strain 26 of S. lactis grown in milk.

Effect of varying amounts of cell-free extract on hydrolysis of peptides

Increasing quantities (up to 0.5 ml.) of cell-free extract of strain 26 of S. lactis were incubated with glycyl-L-leucine and DL-alanylglycine as substrates. As shown in Figure 20B, there appeared to be a direct relationship between the quantity of cell-free extract used and hydrolysis of both peptides, at least up to 0.2 ml. of cell-free extract.

Effect of time of incubation on hydrolysis of peptides

Cell-free extract of strain 26 of S. lactis was incubated with glycyl-L-leucine and DL-alanylglycine as substrates for periods up to 3 hours. Up to at least 1 hour of incubation (Figure 21A), a direct relationship between the time of incubation and amount of hydrolysis of both peptides existed.

Effect of temperature of incubation on hydrolysis of peptides

Cell-free extract (0.2 ml.) of strain 26 of S. lactis was incubated with glycyl-L-leucine and DL-alanylglycine as

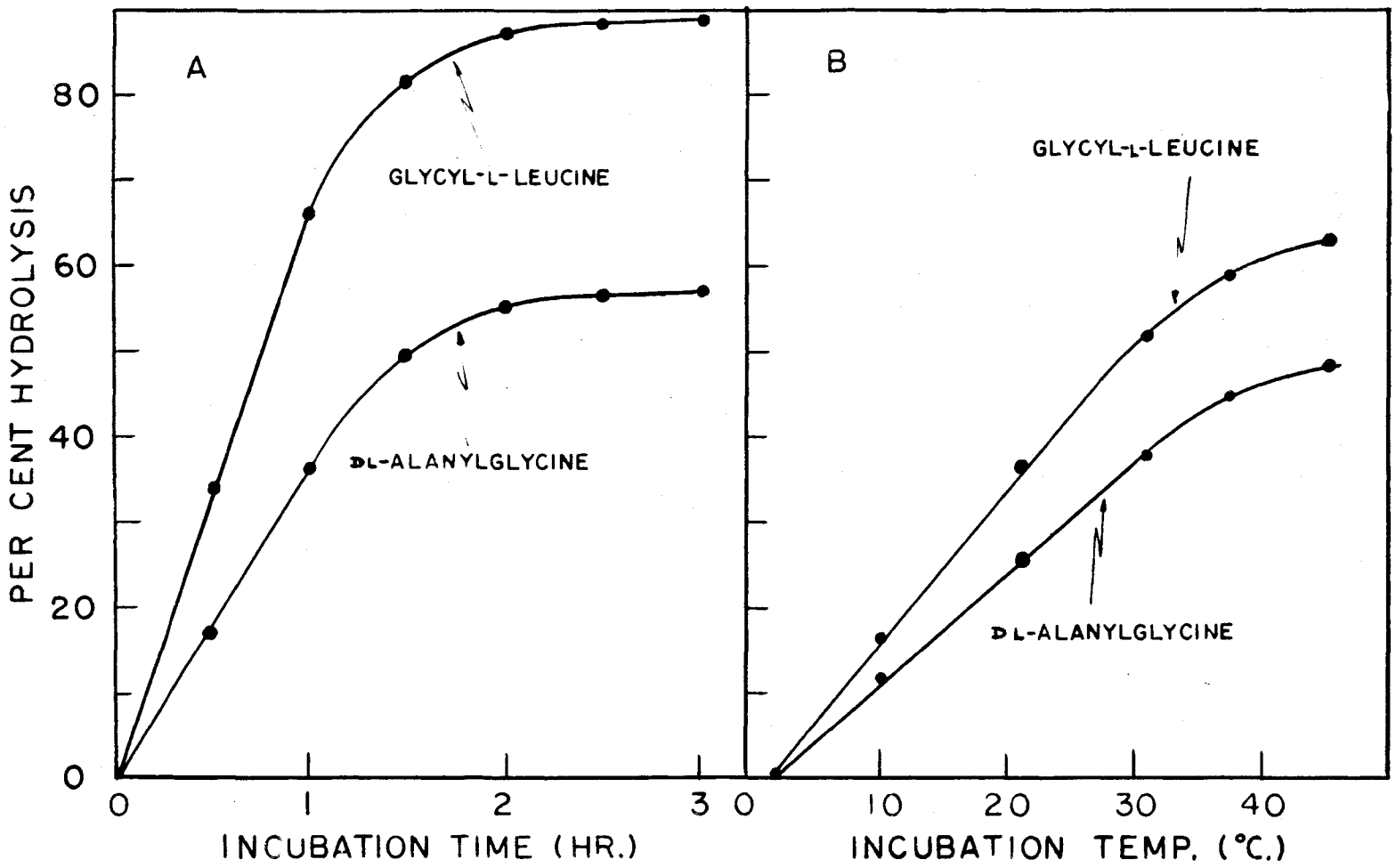

Figure 21. Effect of Time and Temperature of Incubation on Hydrolysis of Peptides by Enzyme Preparation from Strain 26, Grown in Vitamin-Test Casein Medium B. A = 0.2 ml. Enzyme Preparation per 3 ml. Substrate Incubated at 37.5° C. and pH 8.0. B = 0.2 ml. Enzyme Preparation per 3 ml. Substrate Incubated at pH 8.0 for 1 Hour.

substrates at temperatures ranging from 2 to 45° C. Optimum activities against both peptides, within the temperature limits studied, was found at 45° C. (Figure 21B); however, the increase in hydrolysis rate with rising temperatures was somewhat less above 21° C.

The data in Figures 20 and 21, showing the effect of pH, increasing quantities of cell-free extract and time and temperature of incubation on the hydrolysis of peptides by a cell-free extract of strain 26 of S. lactis grown in vitamin-test casein medium B parallel closely those obtained with extracts from cells grown in milk (Figures 15, 16, 17, 18, and 19).

Effect of heating the enzyme preparation on hydrolysis of peptides

Preliminary experiments showed that a cell-free extract of S. lactis could be heated at 55° C. for 30 minutes at pH 7.0 without loss of activity against the different peptides used in this study when tested at pH 7.0, whereas the proteinase activity was almost completely destroyed by heating at 55° C. for 15 minutes (Table 11). Portions of the cell-free extract were adjusted to pH values ranging from 4.0 to 9.0 and heated at 61.7° C. for different lengths of time as described in a previous experiment (pages 80 and 81). The extracts then were tested against glycyl-L-leucine and DL-alanylglycine; incubation was at pH 7.0, because both

peptidase and proteinase activities were determined. Maximum stability of the peptidase activity (Table 16) occurred at pH 7.0. Heating at 61.7° C. at pH 4.0 for only 1 minute destroyed the peptidase activities. Considerable destruction of activities against both peptides took place when heated for more than 3 minutes at pH levels of 5.0 and 9.0.

Table 16.

Effect of Heating at 61.7° C. at Different pH Levels on the Peptidase Activity of a Cell-Free Extract of S. lactis 26[a]

pH of cell-free extract	Control	Per cent hydrolysis after heating for:			
		1 min.	3 min.	5 min.	10 min.
		Glycyl-L-leucine			
4		0	0	0	0
5		62	48	36	30
6		70	68	62	56
7	74	74	70	70	68
8		70	65	52	48
9		67	51	32	28
		DL-alanylglycine			
4		0	0	0	0
5		52	42	25	20
6		56	45	40	43
7	60	62	62	60	58
8		66	51	40	39
9		56	42	35	20

[a] 0.2 ml. enzyme preparation per 3 ml. substrate incubated at 37.5° C. and pH 7.0 for 1 hour.

Stability of the peptidase activities when held at different pH levels at 2° C.

Cell-free extracts of strain 26 of S. lactis were adjusted to pH values ranging from 4.0 to 9.0 and stored at 2° C. for various lengths of time up to 4 days. Following storage, the activity was determined at pH 8.0 against glycyl-L-leucine and DL-alanylglycine. The peptidase activities (Table 17) appeared to be quite stable between pH 6.0 and 9.0. Considerable destruction of activities was observed when stored for 4 days at 2° C. and pH 5.0; holding at pH 4.0 for 12 hours destroyed the peptidase activities completely.

Effect of metallic ions and cysteine on hydrolysis of peptides

The following solutions in 0.01, 0.001 and 0.0001 M concentrations were used: $MnSO_4 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, NiCl, and $CoSO_4 \cdot 7H_2O$. Cell-free extracts of strain 26 of S. lactis (0.2 ml.) were incubated with 1 ml. of the metal solution for 1 hour at 37.5° C., followed by incubation with peptide substrate for 1 hour at 37.5° C. and pH 8.0. As Table 18 shows, when glycyl-L-leucine was used as substrate there was increased activity at all three concentrations of Mn^{++} tested, Cu^{++}, Zn^{++} and Ni^{++} were inhibitory in all three concentrations, whereas Mg^{++}, Co^{++} and cysteine did not show any great effect. When

Table 17.

Stability of Peptidase Activity at Different pH Levels when Held at 2° C. for Various Lengths of Time[a]

pH of cell-free extract (strain 26)	Per cent hydrolysis after holding for:			
	12 hr.	24 hr.	48 hr.	96 hr.
	Glycyl-L-leucine[b]			
4	0	0	0	0
5	50	50	42	30
6	60	50	46	42
7	70	60	50	45
8	72	65	65	60
9	60	62	60	50
	DL-alanylglycine[c]			
4	0	0	0	0
5	50	52	45	32
6	62	60	60	50
7	70	65	62	50
8	70	70	72	60
9	60	62	60	52

[a] 0.2 ml. enzyme preparation per 3 ml. substrate incubated at 37.5° C. and pH 8.0 for 1 hour.

[b] Hydrolysis without holding at 2° C = 72 per cent.

[c] Hydrolysis without holding at 2° C = 74 per cent.

Table 18.

Effect of Metallic Ions and Cysteine on the Peptidase Activity of a Cell-Free Extract of *S. lactis* 26[a]

Metal	Per cent hydrolysis when ion was added in concentration of:		
	10^{-2} M	10^{-3} M	10^{-4} M
	Glycyl-L-leucine		
None	52		
Mn^{++}	75	69	61
Cu^{++}	25	32	39
Zn^{++}	10	20	35
Mg^{++}	50	48	50
Ni^{++}	16	31	43
Co^{++}	45	50	55
Cysteine	47	50	52
	DL-alanylglycine		
None	57		
Mn^{++}	55	50	52
Cu^{++}	46	51	56
Zn^{++}	32	50	52
Mg^{++}	67	60	60
Ni^{++}	21	45	50
Co^{++}	71	70	65
Cysteine	50	50	55

[a] 0.2 ml. enzyme preparation per 3 ml. substrate incubated at 37.5° C. and pH 8.0 for 1 hour.

DL-alanylglycine was the substrate, Co^{++} in all three concentrations used increased the activity, and Mg^{++} gave some activation but only in the highest concentration tested. Cu^{++}, Zn^{++} and Ni^{++} showed inhibitory effect in the 0.01 M concentration; Mn^{++} and cysteine did not show much effect on peptidase activity; the latter possibly was slightly inhibitory at the highest concentration tested.

Purification of the Cell-Free Extract

The results obtained in previous sections indicate that there are at least two different proteolytic systems present in a cell-free extract of S. lactis; a proteinase inactivated when heated at 61.7° C. and pH 7.0 for 2 minutes and peptidases which could withstand this heating without appreciable loss of activity. Besides a multiplicity of enzymes, the cell-free extract contains much inactive material. In subsequent experiments, attempts were made to remove inactive endocellular material and separate some of the proteolytic activities from each other. It was found that both proteolytic systems could be precipitated from the cell-free extract with ammonium sulfate in the range of 40 to 75 per cent saturation. The following procedure has been used with some success. For convenience, the procedure has been presented in Figure 22. All steps in this procedure were performed in a cold room at approximately 2° C.

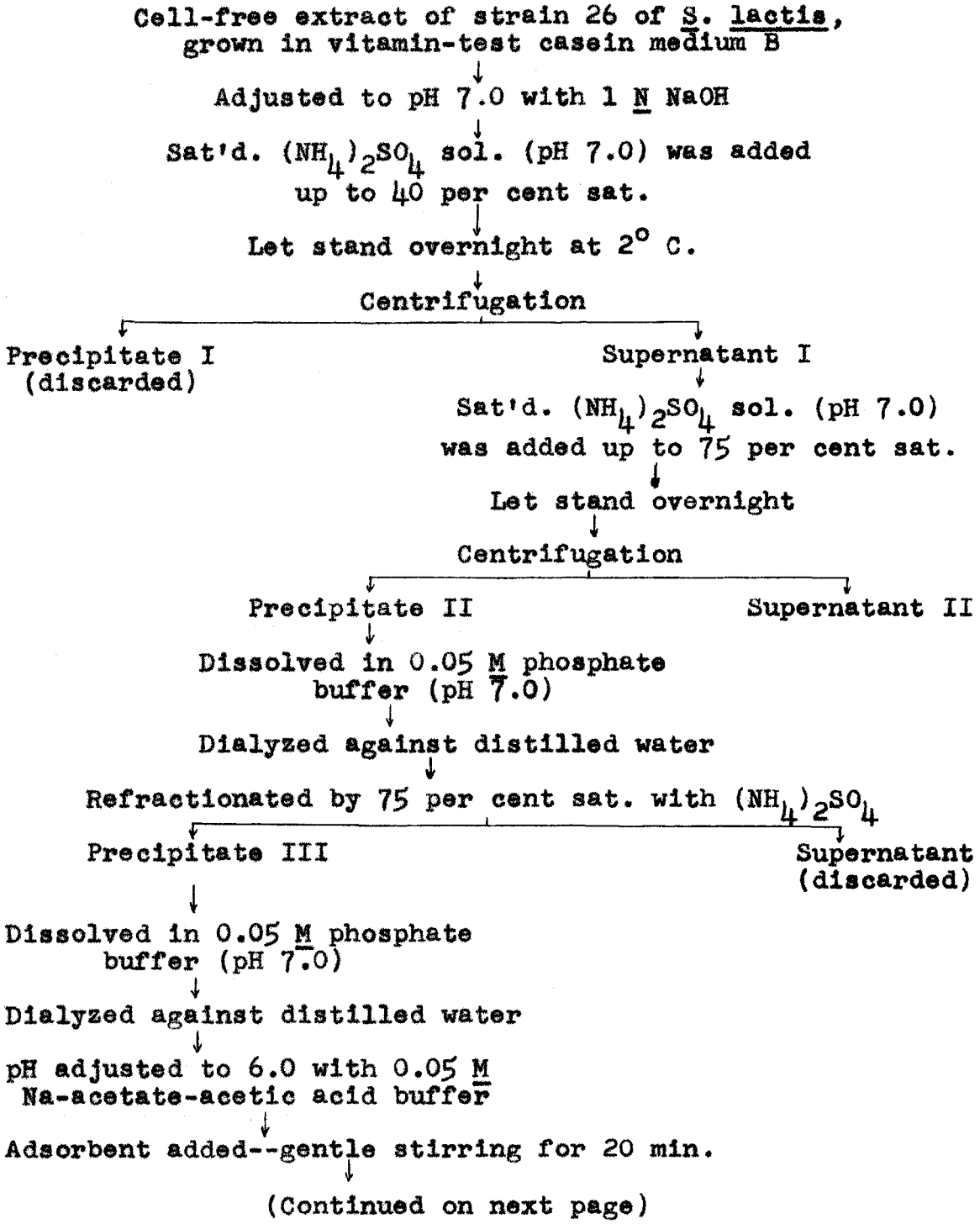

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Figure 22. Fractional Precipitation and Adsorption of Proteolytic Activities.

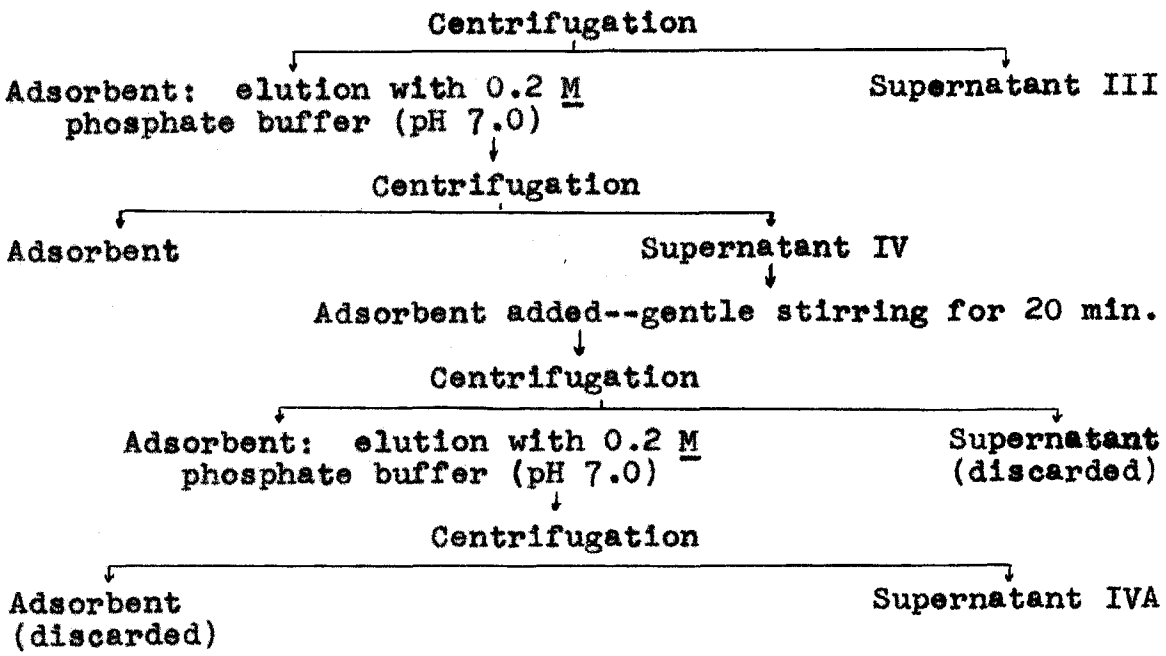

Figure 22. (Continued)

Cell-free extract of strain 26 of S. lactis adjusted to pH 7.0 was saturated up to 40 per cent with ammonium sulfate by adding gradually under stirring a saturated ammonium sulfate solution, which had been adjusted to pH 7.0 with ammonium hydroxide. After standing overnight the precipitate I was removed by centrifugation. Fractions were tested for proteolytic activity against casein, glycyl-L-leucine and DL-alanylglycine as described under Experimental Methods. Precipitate I was in all cases inactive and was discarded. Supernatant I was brought to 75 per cent saturation with respect to ammonium sulfate. After standing overnight, the precipitate II was collected by centrifugation and the inactive supernatant II was discarded. Precipitate II was taken up in a minimum amount of 0.05 M phosphate buffer (pH 7.0), dialyzed against running distilled water for 6 hours and refractionated. Precipitate III was dissolved in a minimum amount of 0.05 M phosphate buffer (pH 7.0), dialyzed as described above and tested for proteolytic activity. The pH then was adjusted to 6.0 with 0.05 M sodium acetate-acetic acid buffer. At this stage an adsorbent was introduced in the procedure, either kaolin or $Al(OH)_3$ Cγ. The preparation of $Al(OH)_3$ Cγ and the purification of commercial kaolin are discussed in the section under Experimental Methods. Either one volume of $Al(OH)_3$ Cγ solution (20 mg. per ml.) was used per 6 volumes of dialyzed material or 25 mg. kaolin per 10 ml. Adsorption was allowed to take place for 20 minutes

under gentle stirring, followed by removal of the adsorbent by centrifugation. The supernatant III was saved and tested. Elution was performed by gentle stirring with 0.2 M phosphate buffer (pH 7.0) for 20 minutes. The adsorbent was removed by centrifugation and the supernatant IV was saved and tested. Supernatant IV was treated with adsorbent as described above, followed by elution, and removal of the adsorbent by centrifugation. Supernatant IVA was saved and tested. Removal of impurities and inert material can be followed by expressing the proteolytic activity in activity per mg. of protein nitrogen present in the material tested. Data in Tables 19 and 20 show that a considerable amount of inactive material could be removed from the cell-free extract by fractional precipitation with ammonium sulfate as is shown, for example, by comparing the proteolytic activity of the cell-free extract and precipitate II per unit protein nitrogen (Tables 19 and 20).

In the adsorption process, both adsorbents showed a preference for the activity against DL-alanylglycine over the one against glycyl-L-leucine; this is demonstrated by the greater activity against DL-alanylglycine per unit protein nitrogen of supernatant IV as compared with supernatant III. The activity against glycyl-L-leucine was divided over both supernatants III and IV. Supernatant IVA did not show any activity against glycyl-L-leucine; this may have been because either the adsorbent showed preference for the

Table 19.

Results of Fractional Precipitation with Ammonium Sulfate and Adsorption with Kaolin

Material tested	Substrate	Proteolytic activity	Protein-nitrogen (mg.)
Cell-free extract	GL[a]	81[c]	1.37
	AG[b]	55[c]	1.37
	casein	65[d]	6.86
Precipitate III	GL	90	0.228
	AG	77	0.228
	casein	46	1.140
Supernatant III	GL	92.5	0.196
	AG	15	0.196
	casein	40	0.392
Supernatant IV	GL	15	0.045
	AG	87.5	0.045
	casein	0	0.225
Supernatant IVA	GL	0	0.04
	AG	75	0.04
	casein	0	0.40

[a] GL = glycyl-L-leucine

[b] AG = DL-alanylglycine

[c] Per cent hydrolysis (incubation at 37.5° C. and pH 8.0 for 1 hour).

[d] γ tyrosine and tryptophan per ml. trichloroacetic acid filtrate (incubation at 37.5° C. and pH 7.0 for 18 hours).

Table 20.

Results of Fractional Precipitation with Ammonium Sulfate and Adsorption with $Al(OH)_3$ Cγ

Material tested	Substrate	Proteolytic activity	Protein-nitrogen (mg.)
Cell-free extract	GL[a]	70[c]	1.12
	AG[b]	48[c]	1.12
	casein	62[d]	5.60
Precipitate III	GL	81	0.179
	AG	50	0.179
	casein	54	0.895
Supernatant III	GL	71	0.154
	AG	12	0.154
	casein	44	0.308
Supernatant IV	GL	3	0.0476
	AG	64	0.0476
	casein	0	0.238
Supernatant IVA	GL	0	0.035
	AG	56	0.035
	casein	0	0.350

[a] GL = glycyl-L-leucine

[b] AG = DL-alanylglycine

[c] Per cent hydrolysis (incubation at 37.5° C. and pH 8.0 for 1 hour).

[d] γ tyrosine and tryptophan per ml. trichloroacetic acid filtrate (incubation at 37.5° C. and pH 7.0 for 18 hours).

activity against DL-alanylglycine or for some other reasons such as inactivation or loss in the process of adsorption and elution. The proteolytic system active against casein was not adsorbed by the adsorbents.

Addition of absolute ethanol to cell-free extract of strain 26 of S. lactis showed that the activities against casein, glycyl-L-leucine and DL-alanylglycine were present in a fraction obtained by increasing the ethanol concentration from 45 to 60 volume per cent; other fractions were inactive against these substrates.

The supernatants III, IV, IVA, and the active fraction obtained by ethanol precipitation were adjusted to pH values ranging from 5.0 to 8.0 and ammonium sulfate was added gradually in steps as to produce 20, 40, 60, and 80 per cent saturation with respect to ammonium sulfate. These attempts to obtain one or more active fractions in crystalline form were not successful. All precipitates were amorphous in nature. Subsequent experiments with supernatant IVA showed that the optimum pH for the activity against DL-alanylglycine was at pH 8.0. The supernatant could be heated for 10 minutes at 61.7° C. (pH 7.0) without loss of activity against DL-alanylglycine; Co^{++} increased the activity. Supernatant III showed optimum activity against glycyl-L-leucine at pH 7.0; heating the extract for 10 minutes at 61.7° C. (pH 7.0) did not affect its activity. Manganous

ions increased the activity against glycyl-L-leucine. These characteristics agree very well with those found for the crude extracts.

Amino Acids and Peptides in the Protein-Free Fraction of Milk with and without Incubation with S. lactis

Little is known about the changes that take place in milk protein as the result of the action of microbial proteinases. In most cases proteolysis is determined by Kjeldahl analysis of the acid-soluble nitrogen, which does not give any information as to the manner in which the changes in the protein take place. In this section the protein-free fraction prepared from skim milk after incubation with S. lactis for different lengths of time without controlled pH was investigated for its content of amino acids in the free and peptide form by paper chromatography.

Milk for these trials consisted of fresh skim milk which was heated for 20 minutes at 185° F., to remove as many undesirable organisms as possible without subjecting the milk constituents to changes that might occur during sterilization. One liter quantities of heated milk were inoculated with 0.1 per cent of a 24-hour culture of S. lactis (strain 26) grown in milk and incubated for 0, 24 and 96 hours at 32° C. Following incubation, a protein-free filtrate was made of each culture by adding trichloroacetic

acid (100 g. per l.milk); the protein was removed by centrifugation and filtration through paper. The amino acids and peptides in the protein-free filtrate were recovered by adsorption and elution from the ion exchange resin Duolite C-3. A portion of the solution containing amino acids and peptides was hydrolyzed with HCl. Two dimensional chromatograms of the unhydrolyzed and hydrolyzed filtrates were carried out. A detailed description of the preparation of the protein-free filtrates for chromatography is presented in the section on chromatography under Experimental Methods.

In Table 21 are presented data showing the amino acids present in unhydrolyzed and hydrolyzed protein-free filtrates of cultures of S. lactis incubated for 0, 24 and 96 hours. Free alanine, glutamic acid, glycine, leucines and valine were present in the protein-free filtrate at 0 hour incubation. Incubation with S. lactis for 24 hours resulted in the appearance in the protein-free filtrate of free lysine, phenylalanine, proline, serine, threonine and tyrosine; an increase was observed in free alanine, glutamic acid, glycine, leucines and valine which already were present at 0 hour incubation. As the period of incubation was extended to 96 hours, free aspartic acid appeared in the protein-free filtrate; increases also were detected in free alanine, glutamic acid, leucines, lysine, phenylalanine, proline, serine, threonine and tyrosine over the amounts found after incubation for 24 hours.

Table 21.

Amino Acids in Unhydrolyzed and Hydrolyzed Protein-Free Fractions of Milk Incubated with S. lactis for 0, 24 and 96 Hours

Amino acid	0 hr.		24 hr.		96 hr.	
	Unhydr.	Hydr.	Unhydr.	Hydr.	Unhydr.	Hydr.
Alanine	+[a]	++[a]	++	+++[a]	+++	+++
Arginine						+
Aspartic acid				+(?)	+	+
Cystine						+
Glutamic acid	+	++	++	+++	+++	++++[a]
Glycine	+	++	++	++	++	++
Leucines	+	++	++	+++	+++	++++
Lysine			+	++	+++	+++
Phenylalanine			+	++	+++	+++
Proline			++	+++	+++	++++
Serine		+	+	++	++	++
Threonine		+	+	++	++	+++
Tyrosine			++	++	+++	+++
Valine	+	++	++	+++	++	+++

[a] + weak, ++ medium, +++ strong, ++++ very strong (on paper).

Hydrolysis of the protein-free filtrate caused in some instances the appearance of amino acids which were not present in the free form before hydrolysis, and caused increases of some of the amino acids present in the free form before hydrolysis. A two dimensional chromatogram representing the amino acids found in a hydrolyzed protein-free filtrate of a culture of S. lactis incubated for 24 hours is presented in Figure 23.

The solution containing amino acids and peptides was fractionated by placing 20 0.01 ml. aliquots equidistant from each other and 1 in. from the bottom of a 12 in. by 16 in. sheet of Whatman no. 1 filter paper. Five sheets were used. These chromatograms were developed using phenol as developing medium in pyrex glass jars with tight fitting lid; each chromatogram was immersed 2 cm. in the solvent, which was allowed to reach the top of the paper. Following development, the sheets were dried in a hood and washed twice with redistilled ether, dried, heated at 100° C. for 15 minutes and viewed under ultraviolet light to locate the amino acids and peptides. The bands were cut out and thoroughly extracted with hot water, concentrated to dryness in vacuo and each residue was dissolved in 1 ml. of 10 per cent aqueous isopropanol. Aliquots of these solutions were hydrolyzed as described earlier. Two dimensional chromatograms were carried out of the unhydrolyzed and hydrolyzed

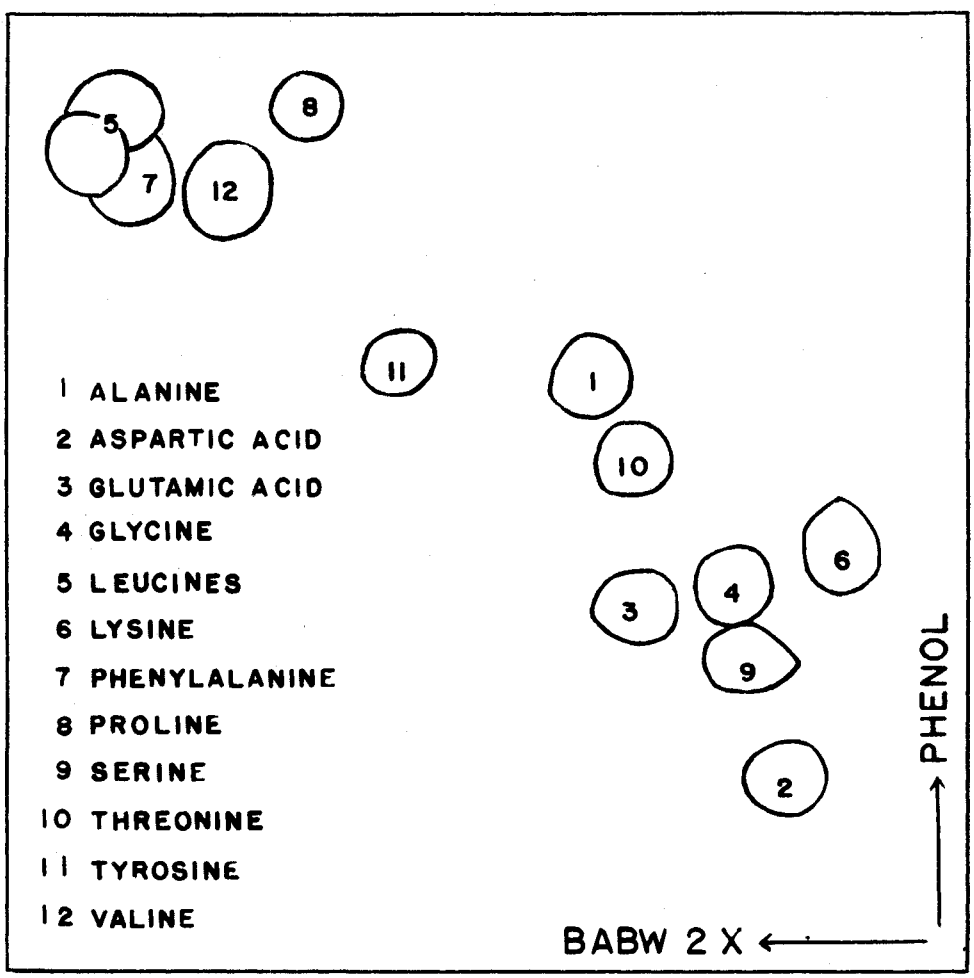

Figure 23. Chromatogram of Hydrolyzed Protein-Free Filtrate of a Culture of S. lactis 26 Incubated at 32° C. for 24 Hours.

materials as described in the section on chromatography under Experimental Methods.

Fractionation showed evidence of at least the following peptides. In the filtrate from the culture at 0 hour incubation: glutamic acid - glycine; alanine - glutamic acid - glycine - leucines; glutamic acid - leucines - proline - serine - valine.

In the filtrate from the culture incubated for 24 hours: alanine - glutamic acid - glycine; glutamic acid - leucines - valine; aspartic acid (?) - glycine - leucines - tyrosine - valine; alanine - leucines - lysine - proline - threonine - tyrosine; alanine - glycine - leucines - phenylalanine - serine.

In the filtrate from the culture incubated for 96 hours: glycine - tyrosine; glycine - leucines - phenylalanine; alanine - lysine - tyrosine - valine; leucines - lysine-proline - threonine; glutamic acid - glycine; glutamic acid - glycine - leucines - proline - valine.

The order in which the amino acids in the peptides are written is arbitrary and does not imply their amounts or structural arrangement in the peptide. Threonine was present in the hydrolyzed aliquot of the protein-free filtrate (0 hour) but not in the peptides, whereas proline was found in one of the peptides and not in the hydrolyzed filtrate. Arginine, aspartic acid, cystine and serine were found in

the hydrolyzed filtrate of a culture that was incubated for 96 hours but these amino acids could not be traced in the peptides of this filtrate.

DISCUSSION

The results obtained in this study seem to indicate that the proteolytic activity of S. lactis is associated closely with the bacterial cell. The marked proteolysis observed in milk cultures of S. lactis during the first 24 hours of incubation would suggest the presence of an enzyme system produced early in the growth phase and excreted in the growth medium. However, attempts to demonstrate proteolytic activity in culture media of various types after the cells had been removed by centrifugation failed. If an enzyme is produced in the early growth phase and excreted in the growth medium, failure to find the enzyme in detectable quantities in the cell-free culture after organism growth might be due to several reasons. First, the enzyme might be unstable or inactive under the conditions used for determination. However, it would be difficult to explain why the enzyme would produce changes in skim milk in the presence of the cells and now show activity when the cells were removed, although in both cases the same substrate (skim milk) was used. A possible factor might be the relative reducing conditions of the medium in presence and absence of actively metabolizing bacterial cells. Secondly, the enzyme might be associated closely with the bacterial

cell. It may be that the enzyme is associated with the surface of the cell and is removed with the cell during centrifugation; this would explain the absence of proteolytic activity in the cell-free culture media. The responsible enzyme system also might be endocellular in the strict sense. Cell-free extracts prepared from S. lactis by sonic vibration showed proteolytic activity against milk, casein, lactalbumin and various peptides. The inability to detect proteolytic activity in culture media after the cells had been removed prevented a direct study of the characteristics of the proteolytic activity associated with the early growth phase in milk. For this reason, the present study has not shown definitely that the proteolytic activity associated with the early growth phase is either identical with or different from that found in a cell-free extract. However, some indication of similarities between the two activities has been found. The greater proteolytic activity in milk cultures of S. lactis at pH levels of 6.0 to 7.5 may be due to greater enzyme production, activity or stability at these levels of reaction. Data on the activity at various pH levels and stability of the proteolytic activity of a cell-free extract of S. lactis indicate that optimum activity and stability occurred at pH values near neutrality. The present work, however, does not enable us to separate definitely production of proteolytic activity from differences in activity at various pH levels.

The patterns of the increases in soluble nitrogen and in tyrosine and tryptophan in milk cultures of S. lactis show a very slight increase in acid-soluble nitrogen during the first 12 hours of incubation, which is in agreement with earlier reports, but definite increases in tyrosine and tryptophan in the protein-free filtrates of these cultures were found within 4 hours. The more rapid early appearance of tyrosine and tryptophan than of increased soluble nitrogen would indicate either that two enzyme systems are involved or that freeing of compounds giving tyrosine and tryptophan reactions with the Folin-Ciocalteau reagent is an early phase of the proteolytic activity. The greater rates of increase in soluble nitrogen at pH 5.5 and, to some degree, at pH 8.0 than of increase in tyrosine and tryptophan at these pH levels indicate the possibility of two systems. Storrs (55) reported increases in tyrosine and tryptophan in protein-free filtrates of milk when a proteolytic enzyme extract (pancreatic) was allowed to react for a short time on this substrate. He attributed the early appearance of tyrosine and tryptophan to changes caused by the enzyme on the surface of the protein molecules, changes that are expected to involve degree of hydration as well as the electrical charge of the particles, properties which are of primary importance with respect to the stability and coagulating characteristics of the protein. Liberation of

tyrosine and tryptophan from milk protein by this pancreatic enzyme was accompanied by decreases in curd tension and protein stability. No other changes in the milk could be observed. Lembke *et al.* (42) recently reported that tyrosine and tryptophan were freed during the early stages of breakdown of milk protein by pepsin and trypsin.

The reaction of the growth medium greatly affected the proteolysis caused by *S. lactis*. As the pH of the system (skim milk) begins to drop and the metabolic activity of the cells presumably begins to decline in the system without maintained pH, the rate of proteolysis, as determined by either procedure, declines. In the samples held at maintained pH levels from 6.0 to 7.5, both determination procedures indicate a continuing proteolysis. Obviously, the decline in proteolysis as pH drops is not due to exhaustion of available substrate, for when the pH is maintained near neutrality substrate continues to be attacked. The possibility exists that the substrate would be not as available for enzyme action at the lower pH levels. Whether or not the pH is maintained, there is little probability that the cells are destroyed by autolysis; although the number of viable cells declines more rapidly in the runs without maintained pH, this need not lead to rapid cell autolysis.

Except for the work by Dudani (20) on the proteinases of *S. liquefaciens*, little attention has been given to the relationship between nutrition and production of this type

of enzyme by the dairy streptococci. In the present work the production of the proteinase activity demonstrable in the cell-free preparation obtained by sonic vibration was influenced to some degree by certain components of the growth medium. This system appears to be partially adaptive, since replacement of whole protein (casein) in the growth medium with breakdown products of proteins caused definite decreases of the proteinase activity. A similar requirement was found by Dudani (20) for the proteinase produced by S. liquefaciens. Zimmerman (60) was not able to demonstrate proteolytic activity against hemoglobin and casein with a cell-free extract of S. cremoris growing in a medium lacking in intact protein.

In the present study, cell-free extracts prepared from S. lactis grown in skim milk showed some indication of a secondary optimum against milk and casein at pH 5.5, but this optimum was not found when the cells were grown in broth media. This may be due to an adaptive enzyme activity responding to some milk constituent or breakdown product not found in the broth media. However, Baribo and Foster (8) reported that a cell-free extract of cells of S. lactis, grown in carrot-liver extract broth showed a second optimum against casein at pH 5.0 to 5.5, using increase in acid-soluble nitrogen as a measure of activity. Differences in the growth media and strains of S. lactis used may be

responsible, at least in part, for this discrepancy. Since the more detailed studies in the present work were made on enzyme preparations from cells grown in non-milk media, data which characterized the system active at the lower pH levels were not obtained.

Presence of nicotinic acid in the growth medium seems to be involved in some manner in production rather than activation of the proteinase activity. Continued growth and production of proteinase activity after omission of one vitamin at a time might be attributed, although very unlikely to (a) the ability of the organism to synthesize these nutrients of (b) the vitamin may not be needed. However, the vitamin-test casein, as usually employed in animal experiments, may contain these compounds in such amounts that growth and production of proteolytic activity, at least in part, continues.

Production of the endocellular peptidases of S. lactis does not seem to be affected by the absence of whole protein (casein) in the growth medium, in contrast to production of endocellular proteinase activity, which was stimulated by the presence of whole protein (casein) in the growth medium. Failure of omission of one vitamin at a time from the medium to influence activity of either of the two peptidases tested for unless growth was affected adversely may be attributed to the same reasons as described for continued

production of proteinase activity after omission of vitamins.

Most of the characteristics of the endocellular proteinase activity of S. lactis, such as effects of pH, temperature of incubation, reducing agents and metallic ions on the hydrolysis of casein agree well with those reported by others (2,8). Baribo and Foster (8), however, found that part of the proteinase activity could withstand heating at 60° C. (pH 5.1) for 30 minutes when the activity was determined against casein at 30° C. In the present study, the activity of the cell-free extract against casein was destroyed by heating at 61.7° C. (pH 5.0) for 1 minute.

In all cases where milk was used as substrate in determining proteinase activity, more proteolytic activity could be demonstrated than with the combined fractions of casein and lactalbumin. The greater proteolysis may have been due to (a) breakdown of one or more non-casein, non-albumin nitrogen fractions of milk or (b) factors inherent in milk which promote the activity of the enzyme system. In addition, in an impure system such as this in which there may be a multiplicity of enzymes, peptidases may act on some of the peptides and be partially responsible for the increased hydrolysis, as demonstrated by determinations of tyrosine and tryptophan. Such activity would not be detected by the soluble nitrogen determination.

There is little opportunity to compare the results on

the characteristics of the endocellular peptidases of S. lactis with the findings of other workers, since little attention has been given to the endocellular peptidases of S. lactis. The activity of a cell-free extract of S. lactis against glycyl-L-leucine was somewhat greater than against DL-alanylglycine, in contrast to the findings of Zimmerman (60) who reported that DL-alanylglycine was split much faster than glycyl-L-leucine. It should be mentioned, however, that the extract used by Zimmerman was prepared from cells of S. cremoris and the cells were grown in a quite different medium from that used in the present study. The effect of metals on hydrolysis of the peptides glycyl-L-leucine and DL-alanylglycine by a cell-free extract of S. lactis are similar to those reported by Zimmerman (60) for a cell-free extract of S. cremoris. The characteristics of the endocellular peptidases of S. lactis integrate well with those reported for a great number of bacteria other than S. lactis by Berger et al. (12).

The differences observed in the effect of pH and metallic ions on the hydrolysis of two or more peptides seem to indicate the presence of different peptidases; this also would be supported by the results of the adsorption experiments which effected a separation of the activities against glycyl-L-leucine and DL-alanylglycine. Although fractional precipitation with ammonium sulfate or ethanol and subsequent adsorption on kaolin or $Al(OH)_3$ Cγ resulted in

separation of the activities against glycyl-L-leucine and DL-alanylglycine, it should be kept in mind that other peptidases still may be present in both fractions. Additional work on fractional precipitation followed by selective adsorption may aid in the separation of the various proteolytic activities of the cell-free extract of S. lactis.

With respect to the results obtained with the various peptides, it should be pointed out that in an impure system such as a cell-free extract of S. lactis, a multiplicity of peptidases probably exists and the action observed against a certain peptide may be the result of the action of several enzymes.

The results obtained in the study of the amino acids and peptides present in a protein-free fraction of milk with and without incubation with S. lactis are difficult to interpret. It is too much to hope that a single study would reveal the pattern followed in the breakdown of milk protein by the proteolytic enzyme system of S. lactis. The free amino acids (alanine, glutamic acid, glycine, leucines and valine) found in the protein-free filtrate of a milk culture of S. lactis at 0 hour incubation also were reported by Block (15) in a protein-free fraction of skim milk. Aspartic acid and serine were reported by Block to be present in very small quantities but were not found in this study at 0 hour incubation. Following incubation with S. lactis

for 4 days at 32° C., increases in the protein-free filtrate in alanine, glutamic acid, glycine, leucines and valine were observed; aspartic acid, lysine, phenylalanine, proline, serine, threonine, and tyrosine also were found in this filtrate. Morgan and Nelson (45) reported also increases in arginine, histidine, methionine, and tryptophan. However, there was considerable difference between the time and temperature of incubation of the milk cultures, preparation of the protein-free filtrates and method of determination of amino acids, used in their study and in the present one. Block (15) reported the presence of at least four peptides; in this study three peptides were reported, of which one was made up from the same amino acids as was one reported by Block (15). The amino acids which occur in the peptides found by Block also were found in the peptides (at 0 hour) in this study with the exception of aspartic acid, which was present in one of the peptides reported by Block. Morgan and Nelson (45) presented evidence that unhydrolyzed filtrates prepared from S. lactis cultures contained intermediate products of protein degradation which possessed amino acid activity. Determination of the order in which the amino acids occur in the peptides found in the various protein-free filtrates undoubtedly would contribute to the knowledge of the mode of action of the proteolytic enzyme system of S. lactis. It seems advisable in future experiments of this type to use a less complex substrate than

milk; the complexity of this substrate increases the difficulties in the interpretation of the results. A similar study employing pure fractions of casein as substrate and purified enzyme extracts probably would eliminate some of the difficulties encountered with milk.

In recent years some attention has been given to the proteolytic activity of water extracts of cheddar cheese (8,49,50). Although some similarity exists between the characteristics of the proteinase activity of cell-free extract of S. lactis, as found in this work, and those reported for cheese extracts (8), such as effect of metallic ions and reducing agents, major differences between these activities were observed. These differences were in the pH values for optimum activity, effect of temperature of incubation and effect of heating on the proteolysis of the substrate. Peterson et al. (49) and Baribo and Foster (8) found optimum activity for cheese proteinase at pH 5.0, with a second optimum near neutrality, whereas the endocellular proteinase of S. lactis in the present study showed optimum activity against casein at pH 7.0. This seems to suggest that a large part of the proteinase extracted from the cheese apparently did not come from S. lactis, otherwise the cheese extract would have been more active near pH 7.0. There are two possible sources of enzymes most active at pH 5.0 to 5.5. One of them is rennet, the other is organisms

with proteolytic enzymes which have lower pH optima. The active proteinase in ripening cheddar cheese was considered by Peterson *et al.* (50) to be largely of bacterial origin on the basis of the increase of cysteine-activated proteinase during ripening. Amundstad(2), however, reported results which he felt showed that rennet causes the greatest part of the breakdown of protein. *L. casei* can not be considered among the organisms with proteolytic enzymes which have lower pH optima since cell-free extracts of this organism showed optimum activity against casein at pH 6.5 (8). Tarnanen (56) reported that cell-free extracts of *Bacterium casei* ε (*L. helveticus*) were most active at approximately pH 6.0 at 42° C. against casein, gelatin and peptone. Assuming that these organisms were present in cheese made of pasteurized milk, it still would be difficult to account for the pH optimum at 5.0 to 5.5. Whether there are other microbial proteinases in milk or cheese with reaction optima at pH 5.0 to 5.5 is not known. In the present study, however, it was found that a somewhat delayed but marked increase in soluble nitrogen occurred in a milk culture of *S. lactis* at pH 5.5. It may be that this component of the proteolytic activity has greater stability at the pH of ripening cheddar cheese than the component active at pH 7.0, and may be part of the proteolytic activity of cheese extracts with optimum at pH 5.0 to 5.5.

The low level of proteinase activity at pH 5.0 found in this study would seem to limit the proteolytic activity of S. lactis in cheese of most common types; the enzyme might be more active on the surface of cheese such as limburger where the pH is raised. However, it should be kept in mind that the breakdown of protein in cheese is the result of the action of enzymes from different sources such as rennet and a variety of microorganisms; each type of organism may contribute a system of proteolytic enzymes. In such case any set of conditions used in the determination of proteolytic activity will select those enzymes which have their optimum activity under these conditions. In addition, the products of the action of one enzyme may act as substrate which would stimulate production of another enzyme. The presence of rennet might give substrates upon which enzymes active at pH 5.0 to 5.5 could be formed and act.

No matter how close the conditions in the test tube experiments approach those prevailing in cheese, extreme caution should be observed in the use of these results to explain changes that take place in cheese during ripening. The conditions that exist in cheese are very complex and undoubtedly differ in many points from those present in the digestion mixtures used in this study. Some of the peptidases present in a cell-free extract of S. lactis proved to be still active at pH values that prevail in cheese of most

common types and could contribute to the hydrolysis of some of the smaller protein fragments.

The inhibitory effect of copper on the hydrolysis of glycyl-L-leucine and DL-alanylglycine is interesting since very small amounts of copper are known to be effective in retarding normal flavor development in cheddar cheese (54).

SUMMARY AND CONCLUSIONS

1. S. lactis when grown in milk caused a rapid increase in both soluble nitrogen and tyrosine and tryptophan during the first 24 hours, followed by a smaller but gradual increase during the rest of the experimental period (70 to 90 hours).

2. The increases in acid-soluble nitrogen during the first 12 hours of incubation were very slight but definite increases in tyrosine and tryptophan in the protein-free filtrates of milk cultures of S. lactis were found within 4 hours.

3. S. lactis grown in milk with maintained pH (5.5 to 8.5) produced at pH values ranging from 6.0 to 7.5 considerably more soluble nitrogen and tyrosine and tryptophan than comparable samples without maintained pH.

4. Addition of calcium carbonate to milk cultures of S. lactis caused in all instances greater proteolysis than in cultures without added calcium carbonate.

5. Lactic acid added to milk to give titratable acidities up to 1.0 per cent, followed by incubation at 32° C. for 24 hours did not cause any increase in either soluble nitrogen or tyrosine and tryptophan, indicating that the protein breakdown in milk cultures of S. lactis is not

due to the lactic acid produced by this organism.

6. No proteolytic activity of either type (increase in soluble nitrogen or in tyrosine and tryptophan) was detected in culture media of various types after the cells of S. lactis had been removed by centrifugation, indicating absence of a true extracellular proteinase.

7. Cell-free extracts prepared from S. lactis grown in either milk or broth media showed proteolytic activity against milk, casein and lactalbumin.

8. Production of proteinase activity of S. lactis appeared to be partially adaptive. Replacement of whole protein (casein) in the growth medium with breakdown products of protein caused definite decreases of the proteinase activity.

9. Omission of individual vitamins from vitamin-test casein medium A affected organism growth. Omission of nicotinic acid from the growth medium caused a definite decrease of the proteinase activity of the cell-free extract. The presence of this vitamin in the growth medium seemed to be involved in some manner in the production rather than activation of the endocellular proteinase activity.

10. The endocellular proteinase activity had its optimum pH against milk and casein at pH 7.0; optimum pH for lactalbumin was at pH 6.5. When milk was used as growth medium some indication of a second optimum against milk and

casein as substrate was found at pH 5.5; this however was not found when the cells were grown in vitamin-test casein medium B.

11. Maximum activity of the endocellular proteinase against α and γ casein was found at pH 6.5, for β casein at pH 6.5 to 7.0. α casein was digested somewhat more than β and γ casein at pH 6.0 to 7.0.

12. Optimum activity of the endocellular proteinase against milk, casein and lactalbumin, within the temperature limits studied, was found at 45° C.

13. Reducing agents activated the endocellular proteinase.

14. Metallic ions either had no effect on the proteinase activity or were slightly inhibitory.

15. The effect of heating at 50, 55 and 61.7° C. for different lengths of time was tested at various pH levels of the cell-free extract. Maximum stability of the proteinase activity was found at pH 7.0. Heating at 61.7° C. for only 2 minutes inactivated the preparation. The extract was inactivated quite rapidly at pH's 5.0 and 9.0, even at the lower temperatures.

16. The proteinase activity was quite stable between pH 5.0 and 9.0 during 4 days of storage at 2° C. A considerable destruction took place at pH 5.0 and 9.0 when stored at 32° C. and a somewhat smaller destruction at pH 6.0 and 8.0 at 32° C.

17. A cell-free extract of S. lactis grown in either milk or broth media was found to be active against seven different peptides.

18. Production of endocellular peptidases active against glycyl-L-leucine and DL-alanylglycine was not affected by the absence of whole protein (casein) in the growth medium, in contrast to the production of the endocellular proteinase activity which was stimulated by the presence of whole protein (casein) in the growth medium.

19. Omission from vitamin-test casein medium A of biotin, nicotinic acid and pyridoxine decreased the activity of the cell-free extract of S. lactis against glycyl-L-leucine somewhat; omission of biotin, nicotinic acid and calcium pantothenate decreased the activity against DL-alanylglycine.

20. The optima for the activities against seven different peptides were found at pH values ranging from 7.0 to 9.0. There was some tendency of the peptides in which the glycine carboxyl group was free to have the pH of maximum activity at a somewhat higher value than the leucine or tyrosine peptides.

21. Optimum activity against glycyl-L-leucine and DL-alanylglycine, within the temperature limits studied, was found at 45° C.

22. The effect of heat on the peptidase activity against glycyl-L-leucine and DL-alanylglycine was tested by

heating the cell-free extract at 61.7° C. for different lengths of time at various pH levels (4.0 to 9.0). Maximum stability of the peptidase activities was at pH 7.0; the extract could withstand heating at 61.7° C. (at pH 7.0) for 10 minutes without any serious loss of activity. Heating at 61.7° C. at pH 4.0 for 1 minute destroyed the peptidase activities. Considerable destruction of activities against both peptides took place when heated for more than 3 minutes at pH's 5.0 and 9.0.

23. The activities against glycyl-L-leucine and DL-alanylglycine were quite stable between pH's 6.0 and 9.0 during 4 days of storage at 2° C. Considerable destruction of activities occurred when stored at pH 5.0 and 2° C. for 4 days. Holding at pH 4.0 for 12 hours destroyed the peptidase activities completely.

24. Hydrolysis of glycyl-L-leucine at pH 8.0 was activated by Mn^{++}; Cu^{++}, Zn^{++} and Ni^{++} were inhibitory, Mg^{++}, Co^{++} and cysteine did not have much effect.

25. In the case of DL-alanylglycine, Co^{++} activated the hydrolysis at pH 8.0, Mg^{++} in 0.1 M concentration gave some activation, Cu^{++}, Zn^{++} and Ni^{++} were inhibitory and Mn^{++} and cysteine did not show much effect.

26. Both the proteinase activity and the peptidase activities against glycyl-L-leucine and DL-alanylglycine were precipitated from a cell-free extract of S. lactis with

ammonium sulfate in the range between 40 and 75 per cent saturation, and also by absolute ethanol between 40 and 60 volume per cent at pH 7.0.

27. Fractional precipitation and adsorption experiments removed a great portion of the inactive material from the cell-free extract and resulted in some separation of the activity against DL-alanylglycine from that against glycyl-L-leucine and from the proteinase activity.

28. The protein-free fraction of milk incubated with S. lactis for 0, 24 and 96 hours was investigated for the presence of amino acids and peptides by paper chromatography. Free alanine, glutamic acid, glycine, leucines and valine were present in the protein-free filtrate at 0 hour incubation.

29. Incubation with S. lactis for 24 hours resulted in the appearance of free lysine, phenylalanine, proline, serine, threonine and tyrosine; an increase was observed in free alanine, glutamic acid, glycine, leucines and valine, which already were present at 0 hour incubation.

30. As the incubation period was extended to 96 hours, free aspartic acid appeared; increases also occurred in free alanine, glutamic acid, leucines, lysine, phenylalanine, proline, serine, threonine and tyrosine over the amounts found after incubation for 24 hours.

31. Evidence was found for the presence in the

protein-free filtrate of three peptides at 0 hour, five peptides after 24 hours of incubation and six peptides after 96 hours of incubation.

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