OBSERVATIONS ON THE PROTEOLYTIC ACTIVITY OF CRUDE EXTRACTS OF *LACTOBACILLUS CASEI*

By

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TABLE OF CONTENTS

		Page
I	HISTORICAL INTRODUCTION	1
II	EXPERIMENTAL	17
	A. Cultures	17
	B. Determination of proteolytic activity of ten strains of *Lactobacillus casei*	19
	C. Determination of soluble nitrogen in cultures	22
	D. Preparation of mass cultures of *Lactobacillus casei* 318	23
	E. Methods used to prepare crude cell-free extracts	23
	F. Analytical methods	34
III	RESULTS	
	A. Proteolytic activity of ten strains of *Lactobacillus casei*	37
	B. Mass culture of *Lactobacillus casei* 318	37
	C. Relative effectiveness of methods used to prepare cell-free extracts	39
	D. Action of extracts prepared in Mickle Tissue Disintegrator	70
	1. Disruption of cells	70
	2. Determination of optimum shaking time	71
	3. Activity of extract against gelatin	74
	4. Influence of glutathione on the hydrolysis of casein	74
	5. Influence of various metallic ions	76
	6. Activity of extract against peptones	81
	7. Activity of extract against peptides	82
IV	DISCUSSION AND SUMMARY	87
V	LITERATURE CITED	96

LIST OF TABLES

Table		Page
1.	Number and history of 14 strains of Lactobacillus casei.	18
2.	Composition of calcium carbonate-skim milk cultures used to determine casein hydrolysis.	20
3.	Plate counts of calcium carbonate-skim milk cultures of Lactobacillus casei.	21
4.	Nitrogen determinations of 12 week old cultures of 10 strains of Lactobacillus casei.	38
5.	Tryptic activity of extract from sand ground cells.	40
6.	Tryptic activity of extract prepared by alternate freezing and thawing.	40
7.	Polypeptidase activity of extract obtained by grinding with alumina.	42
8.	Caseolytic activity of extract obtained by grinding with alumina.	44
9.	Gelatinolytic activity of extract obtained by grinding with alumina.	45
10.	Proteolytic activity of extract obtained by extracting alumina-cell mass with water.	46
11.	Proteolytic activity of extract obtained by extracting alumina-cell mass with M/15 phosphate solution.	47
12.	Proteolytic activity of extract obtained by grinding with Alcoa E 20.	49
13.	Proteolytic activity of glycerol extract using casein and dl-leucylglycine as substrates.	50
14.	Proteolytic activity of glycerol extract using several types of peptones as substrates.	51
15.	Caseolytic activity of extract obtained by shaking cells with 5 mm glass beads at pH 8.9 for 2 and 4 hours.	53
16.	Proteolytic activity of extract obtained by shaking cells with 5 mm glass beads at pH 8.9 for 4 hours using peptones as substrates.	54

Table | Page

17. Dipeptidase activity of extract obtained by shaking cells with 5 mm glass beads for 2 and 4 hours using M/15 dl-leucylglycine as substrate. - - - - - - - - - - - - - - - - - 55

18. Caseolytic activity of extract obtained by shaking cells with 5 mm glass beads at pH 6.6 and pH 7.0. - - - - - - - - - 57

19. Caseolytic activity of extract obtained by shaking cells with 5 mm glass beads at pH 5.8. - - - - - - - - - - - - - - - - 58

20. Proteolytic activity of extract obtained by shaking cells with 5 mm glass beads at pH 5.8 for 4 hours using several types of peptones as substrates. - - - - - - - - - - - 59

21. Caseolytic activity of extract prepared by shaking cells with small glass beads for 2 and 4 hours. - - - - - - - - - - 60

22. Caseolytic activity of extracts prepared by exposing cells to ultra-high-frequency vibrations. - - - - - - - - - - - - - 62

23. Proteolytic activity of extracts obtained after autolysis of cells at pH 7.3 for 2 weeks. - - - - - - - - - - - - - - - 64

24. Proteolytic activity of extracts obtained after autolysis of cells with Tween 80 at pH 4.5 for 2 weeks. - - - - - - - - 65

25. Proteolytic activity of extracts obtained after autolysis of cells with Tween 80 at pH 8.9 for 2 weeks. - - - - - - - - 65

26. Caseolytic activity of extracts prepared by treating cells with glycine at 30 C and 6 C for 24 hours. - - - - - - - - - 67

27. Caseolytic activity of extract prepared by treating cells with rennin at pH 5.0. - 69

28. Caseolytic activity of extracts prepared in Mickle Tissue Disintegrator at pH 5.8 and pH 8.9. - - - - - - - - - - - - - 75

29. Gelatinolytic activity of extracts at various pH values. - - - - 76

30. Caseolytic activity of untreated and glutathione treated extract. - 77

31. Influence of various inorganic ions on the relative activation or inhibition of caseolytic activity of extract. - 79

Table Page

32. Influence of various inorganic ions on the relative activation or inhibition of gelatinolytic activity of extract. - 80

33. Proteolytic activity of extract using several peptones as substrates at different pH values. - - - - - - - - - - - - - 81

34. Determination of dipeptidase activity of extract using dl-alanylglycine and d-leucyl-l-tyrosine. - - - - - - - - - - 84

35. Determination of dipeptidase activity of extract using glycyl-l-leucine and l-leucylglycine. - - - - - - - - - - - - 85

LIST OF FIGURES

Figure		Page
1.	Mickle Tissue Disintegrator. - - - - - - - - - - - - - - - - - -	33
2.	Cells of Lactobacillus casei 318 before shaking in the Mickle Tissue Disintegrator. X 970 - - - - - - - -	72
3.	Cells of Lactobacillus casei 318 after shaking for 20 minutes in the Mickle Tissue Disintegrator. X 970 -	72
4.	Electronograph of Lactobacillus casei 318 suspension before shaking. X 10,000 -	73
5.	Electronograph of Lactobacillus casei 318 after shaking for 20 minutes in the Mickle Tissue Disintegrator. X 10,000 -	73

I

HISTORICAL INTRODUCTION

The enzymes which are capable of catalyzing the hydrolysis of proteins, proteoses, peptones, and polypeptides are called proteases. Proteases are further classified into proteinases and peptidases. The proteinases are considered to be capable of splitting proteins as well as the proteoses and peptones, whereas the peptidases are assumed to cause the hydrolysis of peptides with the formation of amino acids. Thus, according to Grassmann and Schneider (1936), peptidases are classified into dipeptidases, aminopolypeptidases and carboxypolypeptidases. Dipeptidases hydrolyze dipeptides of amino acids. Aminopolypeptidases hydrolyze polypeptides adjacent to the points where the free amino groups are found. If the amino group is blocked or is found on a d-amino acid residue, then there is no enzymatic action. Carboxypolypeptidases will attack polypeptides having a free carboxyl group near the peptide linkage being attacked. A blocked amino group will not interfere with the activity of this enzyme, but a blocked carboxyl group here, will prevent this enzyme from acting on its substrate.

Proteolytic activities of plant and animal tissues and secretions have been known for at least 150 years, since Spallanzani in 1783 first observed the digestion of meat in vitro by gastric juice. (Northrop, et. al., 1948).

The proteolytic activities of many different types of bacteria have been the subject of study for a long period of time. According to Porter (1946), the first controlled experiments on the proteolytic activities of bacteria were performed in 1886. Since that time a very extensive literature has been published relative to this phase of bacterial physiology. However, much of the early work in this field was of an indecisive and conflicting nature. Recently, with the introduction of chemically defined substrates more accurate and decisive information concerning the proteolytic enzymes of bacteria has been obtained.

A perusal of the literature indicates that most of the definitive studies on bacterial proteases have been made during the last twenty years. Buchanan and Fulmer (1930) have presented an extensive review of much of the important early work accomplished in this field prior to 1930. The review of the literature presented here therefore, will be confined to the work published since that time.

Gorini (1930), reported his findings on the milk clotting power of Bacillus prodigiosus. He stated that a rennin-like enzyme was produced by this bacterium. The production of this enzyme took place in various types of media in the absence of casein and lactose. The bacterial rennin was capable of acting on heated and/or autoclaved milk, and could be destroyed by heating to 100 C for 15 minutes. Wilson (1930) studied the elaboration of bacterial protease in media of various composition. She found that the carbon source had no effect on the appearance of a protease and that the enzymes may be elaborated as readily in the presence of inorganic nitrogen sources as with organic nitrogenous compounds. It was concluded that protease production was not dependent

upon the ingredients of the nutrient medium except as these stimulate or inhibit growth of the cells. Haines (1931; 1932; 1933), working with several organisms showed that calcium and magnesium affected the activity of gelatinase production. He noted that their effect was not due to activation after the enzyme was formed. Gorbach (1930), studied the proteases of Bacillus pyocyaneus which were produced during the growth of the organism in various media. The proteolytic activity of cell-free filtrates was determined using various substrates such as gelatin, Witte-peptone, silk peptone and albumin peptone. The peptides used were leucylglycine, and leucylglycylglycine. It was found that the preparation obtained was a complex mixture of enzymes acting on the different substrates having various pH optima. Virtanen and Tarnanen (1932), studied the heat resistance of a filtrable proteinase produced by a strain of B. fluorescens liquefaciens (Pseudomonas fluorescens). They found that the proteinases obtained when the organism was grown in broth or milk retained about 72 per cent of their activity when heated for ten minutes at 90 C. The proteinases were inactivated when they were heated at 115 C for ten minutes. Tarr (1934), studied the proteolytic activity of spore suspensions of Bacillus subtilis and Bacillus mesentericus. Suspensions of spores of these organisms were heated for thirty minutes at 80 C. The heated bacterial endospores hydrolyzed casein, gelatin, egg-albumin, blood-albumin, Witte's peptone, and in the case of the spores of Bacillus subtilis, edestin. The hydrolysis was carried out at pH 7.3 for two days at 37 C. Weil and Kocholaty (1937) studied the cell-free filtrate from cultures of Clostridium histolyticum. The proteinase of this anaerobe, showed maximum activity at pH 7.0 and was activated by sulfhydryl compounds, but not by enterokinase. The activation by sulfhydryl compounds could

be increased by the presence of ferrous, manganous, nickle, cupric, and cobalt ions. All of these ions except cobalt produced activation at very low concentrations. Maximum activation however, was obtained under anaerobic conditions. It was found that iodoacetic acid did not inhibit the proteinase activity. Grassmann (1938), studied the effects of normal horse serum on proteinases of several clostridia. He reported that inhibition of the proteinase of Clostridium botulinum was observed. However, normal horse serum apparently did not inhibit the hydrolytic activity of the proteinase from cultures of Clostridium welchii. The proteolytic enzymes of the latter organism were however, inhibited by a specific immune serum prepared against Clostridium welchii. Maschmann (1938), reported on the proteinases of Clostridium histolyticum. This worker described three distinct proteinases, two of which he considered to be extracellular and the third he considered to be intracellular. The extracellular enzymes were found to be inhibited by cysteine and ferrous ions. However, the intracellular enzyme differed from the others in being able to hydrolyze clupein but not gelatin and was activated by sulfhydryl compounds. This report contradicted Kocholaty, Weil, and Smith (1938), who reported that the proteinase of Clostridium histolyticum is liberated into the culture medium by secretion from the living cell. They reported also that the enzyme is activated by cysteine and ferrous ions under all conditions. Kocholaty, Smith, and Weil (1938), investigated the peptidases of Clostridium histolyticum. They obtained enzymatically active cell-free solutions by disrupting the bacterial cells with the supersonic

oscillator, The cell-free solutions which they obtained contained a dipeptidase with an optimum activity of pH 7.6 and a polypeptidase which acted optimally at pH 8.7. It was found that the polypeptidase was activated by magnesium ions but that this ion had no effect on the activity of the dipeptidase. Kocholaty and Weil (1938), found also that by changing the pH of the culture medium in which Clostridium histolyticum was growing, the pH optimum of the secreted proteinase could also be changed. By repeated culturing of the organism on media containing either casein or gelatin, a proteinase was produced which would only attack casein or gelatin.

Berger, Johnson, and Peterson (1938a), studied extensively the peptidases of Leuconostoc mesenteroides. They obtained an enzymatically active extract by a combination of methods for disrupting the bacterial cells. The cells of Leuconostoc mesenteroides were alternately frozen and thawed in the refrigerator for six days. Following this they were ground with sand and then allowed to autolyse under toluene at room temperature, at pH 6.8-7.0. In such a cell free autolysate, peptidases capable of hydrolysing both optical components of the racemic peptides, leucylglycine, leucyldiglycine, alanylglycine and alanyldiglycine were found. In further studies Berger, Johnson, and Peterson (1938b), reported on the peptidases of some common bacteria. The peptidases obtained from twelve different organisms representing aerobic, anaerobic, facultative, proteolytic and non-proteolytic bacteria were studied. The cell-free bacterial extracts were prepared according to the method which had been described in their previous paper. They found that in

most instances the optimum value for peptide hydrolysis was between pH 8-9. There were however, two organisms namely, Lactobacillus pentosus and Propionibacterium pentosaceum which contained acidopeptidases. These enzymes split their substrates at optimum pH values of 5.5 to 6.0. Generally, it was found that more peptidases could be extracted from the cells of non-proteolytic organisms than from the medium in which the cells were grown.

Johnson (1938), presented a review of the information concerning proteinases and peptidases of microorganisms. He stated that almost any known type of peptidase system has been found among the bacteria, and also that a number of previously unknown types of peptidases have also been observed and reported on. The bacterial decomposition of gelatin was studied by Console and Rahn (1938). They found that the decomposition was partly due to an extracellular enzyme and that there was no definite relationship between the number of cells present and the amount of decomposed gelatin. The addition of glucose at the beginning of the experiments prevented almost entirely the formation of proteolytic enzymes. These workers however, found that if glucose was added to a culture which was seven days old, the enzyme formation was not prevented although acid formed from the added glucose was found to retard the rate of enzyme action.

Ålberg and Meyer (1939), studied the nature and properties of the various proteolytic enzymes produced by Clostridium botulinum and their relationship to toxin production. They found that a proteinase acting optimally at pH 7 on gelatin and casein was secreted into the

medium by the organism. This proteinase was inactivated by sodium cyanide, copper salts, and hydrogen peroxide. In addition, it was shown that a pdypeptidase, aminopolypeptidase and a dipeptidase, all acting optimally at pH 7.8 to 8.0 were produced during growth of the organism and were secreted into the medium. The proteinase was found in the culture medium approximately six hours before the botulinus toxin could be demonstrated. On the other hand, it was found that the polypeptidases and dipeptidase appeared simultaneously with the toxin. The inhibition of proteinases of certain members of the genus Clostridium was studied by Smith and Lindsley (1939). The proteolytic activity of culture filtrates of various clostridia was tested using gelatin as the protein substrate. The gelatinolytic ability was found to be inhibited for Clostridium aerofetidum, Clostridium botulinum, Clostridium fallax, Clostridium putrificum, Clostridium sporogenes and a rough strain of Clostridium histolyticum by albumin and beta-globulin of normal rabbit serum.

Weil, Kocholaty and Smith (1939), reported on the proteinases of five species of the genus Clostridium and four aerobes. They found that all of the clostridia studied secreted an extracellular proteinase which acted on gelatin and was maximally activated by a combination of cysteine and ferrous ions. The proteinases of the aerobes on the other hand, were partially inhibited by cysteine, although marked activations were discovered when cysteine in combination with ferrous ions was used. Optimal activity of all the proteinases studied occurred at pH 7.0.

Ayres and Tobie (1943), reviewed the proteolytic activities of

a large number of strains of molds in the genera Aspergillus and Penicillium and bacteria in the genera Pseudomonas, Clostridium, and Bacillus. These authors state that molds and bacteria which are capable of producing proteolytic enzymes usually give very active solutions of proteases when bran cultures of molds are extracted with water or dilute saline, or when liquid cultures of bacteria are tested directly. These workers also state that properly prepared dried preparations of these enzymatically active solutions give solid concentrates which are more active against casein than is U.S.P. pancreatin.

Elliott (1945), detected a proteolytic enzyme in the cell-free filtrate of broth cultures of certain group A streptococci which attacked the type-specific M antigens of the organisms. This enzyme was most active at 37 C and no M substance could be produced from enzyme-producing cultures which had been grown at this temperature. In addition to attacking the streptococcal M substance, the proteinase attacked human and rabbit fibrin, casein, milk, gelatin, and benzoyl-l-arginineamide. It did not attack l-leucylglycylglycine. The enzyme resembled papain and some of the cathepsins since it was activated by potassium cyanide cysteine, glutathione, and thioglycollic acid, but was not activated by ascorbic acid. The enzyme was inactivated by iodoacetic acid and also by normal rabbit and mouse sera. Roulet and Zeller (1945), showed that the organism Mycobacterium tuberculosis produced peptidases which could hydrolyze glycyl-l-tyrosine, glycyl-l-leucine, l-leucylglycine and l-leucylglycylglycine. All of these enzymes were found to be activated by very low concentrations of manganese ions.

Stockton and Wyss (1946), studied certain food spoilage organisms, and observed that Bacillus subtilis did not produce proteinases in aerated nutrient broths containing Difco peptone and beef extracts. However, the same organism demonstrated a good proteinase production when grown in media containing Armour's or Witte's peptone. This discrepancy of proteinase production was found to be due to mineral deficiency, since a substantial increase in proteinase formation was obtained in Difco broth by the addition of 0.05 to 50 mg. of manganese sulphate per liter. The added manganese compound appeared to function during the elaboration of the enzyme and apparently did not act merely as an activator of the proteolytic enzyme system. Slightly greater enzyme production was noted when a mixture of manganese ions and zinc ions were used instead of manganese ions alone.

Chopra (1946a), studied the filterable proteinases produced by some thermophilic bacteria. He found that the proteinases of Bacillus thermophilus, Bacillus aerothermophilus, and Bacillus thermoacidurans, hydrolysed gelatin and casein readily at an optimum pH 8.0. Albumin was acted on only after previous denaturation and at an alkaline pH. These proteinases therefore, were assumed to resemble "tryptases." In addition to proteinases, the thermophilic bacteria studied produced a polypeptidase capable of hydrolysing peptone. In several other papers Chopra (1946b,c), it was reported that the proteinases of the thermophilic organisms studied appeared to be endocellular, and were found in culture filtrates after the organisms underwent autholysis. It seemed that filtrates which contained calcium and magnesium ions were more active proteolytically

than filtrates which were free of these ions. Copper ions had no effect on these proteinases but their activity was suppressed by methylene blue and inhibited by iodine. The activation mechanism and physico-chemical properties of a purified preparation of Clostridium histolyticum were reported on by Kocholaty and Krejci (1948). These workers reported that a homogeneous protein was obtained from culture of the organism by a combination of precipitation and electrophoretic procedures in the Tiselius apparatus. It was found that the purified enzyme preparation had a limited range of hydrolysis on gelatin and clupein which could be increased by the addition of ferrous ions and sulfhydryl compounds. The mechanism of activation appeared to depend upon the formation by the iron and cysteine of an addition compound with the enzyme.

Bidwell (1949), studied certain properties of K-toxin (collagenases) of Clostridium welchii. She found that the true collagenase, an enzyme capable of disintegrating muscle and collagen fiber, was destroyed by exposure to a low temperature at pH 9 to 10. It was also found that the collagenase was destroyed by exposure to 50 C at a more acid pH, than that indicated above. However, these treatments released another enzyme which attacked hide powder and azocoll. This second enzyme was destroyed either by heating to 60 C for ten minutes or by exposure to a pH greater than 10.5.

Gorini and Framageot (1949), described a proteinase from Micrococcus lysodeikticus which did not hydrolyse any of the protein substrates used, in the absence of calcium ions. These workers also found

no evidence of activation or inhibition when sulfhydryl group compounds such as glutathione or cysteine were used. Virtanen and Winkler (1949), found that the proteolytic enzyme system of Escherichia coli was not influenced by a pronounced decrease in the amount of available nitrogen present in the medium in which the organism was growing. Jones, Stacey and Webb (1949), reported on an autolytic enzyme system present in gram positive bacteria. It was found by these workers that the first step in the process of cell autolysis resulted in the destruction of the characteristic gram stain reaction. This effect was found to be due to ribonuclease activity. The second step of the process involved the decomposition of the cystoskeleton, which had already been rendered gram negative by the ribonuclease action. This action was the result of a proteolytic system composed of two enzymes. One of these enzymes was capable of hydrolysing casein but not peptone, while the other was found capable of hydrolysing only peptone.

Elliot (1950), found that some strains of group A streptococci elaborated the precursor of a proteolytic enzyme when grown in a dialysate broth adjusted to pH 5.5 to 6.5. It was found that the proteinase precursor could be crystallized from half-saturated ammonium sulfate solution at a pH 8.8 and a temperature of 22 C or higher. On the other hand it was found that the proteinase crystallized from 0.15 saturated ammonium sulfate solution at pH 8.0 and at refrigerator temperature. Immunological studies showed evidence of distinct antigenic specificity of the precursor and the proteinase.

Bacteria which are capable of attacking the sugar lactose to

produce relatively large amounts of lactic acid are called characteristically the "lactic acid bacteria." Chief among these are the genera Streptococcus and Lactobacillus. Although these organisms are considered to be primarily saccharolytic, as a predominant biochemical characteristic, they have nevertheless, the ability of causing the breakdown of proteins under certain conditions. Empirically, evidence for the proteolytic activity of the lactic acid bacteria is found in their association with the protein material during the period of proteolysis which constitutes the ripening process of cheese.

As early as 1891, von Freudenreich published a theory explaining the ripening process of Emmenthaler cheese as one involving the splitting of the casein in the curd by the action of certain bacteria found in the curd. In an extensive study, von Freudenreich (1895, 1897, 1899), showed that the most important organisms found in the ripening casein curd of Emmenthaler cheese were the rod-shaped lactic acid bacteria. These studies were extended by von Freudenreich and Orla-Jensen (1900). The organisms which were thought most important in the ripening process by these workers, became known as Bacterium casei epsilon. Although Orla-Jensen found these organisms capable of very extensive saccharolytic activity, he nevertheless demonstrated that when the organisms were grown in sterile skim milk in the presence of sterile calcium carbonate, the non-protein amino nitrogen of the culture medium was greatly increased. Summing up this facet of his researches into the nature of the rod-shaped bacteria found in cheese, Orla-Jensen (1919), wrote .....

> ..... the proteolysis produced by lactic acid bacteria is most active in old cultures with many dead cells. There is no doubt that the hydrolysis of proteins, like that of the sugars, is due to endo-enzymes, and does not therefore spread in the nutritive substrate until the cells have become weakened, or even autolysed. It is the proteolytic enzymes of the lactic acid bacteria which occasion the ripening of cheese, and this process only reaches distinct development long after the sugar in the cheese has been fermented, when the lactic acid bacteria have ceased their activity, being either dead or in a latent state. The fact that lactic acid bacteria only decompose proteins to amino-acids, but do not decompose the latter any further makes the maturing of cheese equal to the process of digestion and not to that of putrifaction, which is particularly characterized by a breaking down of amino-acids.

Barthel (1916), in his study of the proteolytic activities of both the lactic acid streptococci as well as Bacterium casei epsilon, found that they were capable of causing the hydrolysis of casein. However, it was found that the splitting of casein by Bacterium casei epsilon proceeded only when the culture was incubated at 37 C. No hydrolysis of the casein substrate could be demonstrated when the culture was held at room temperature. Other studies on the relationship of proteolysis by Bacterium casei epsilon and the temperature requirements were published by Virtanen, Wichman, and Lindstrom (1927a, b). The proteolytic activity

of twenty-two strains of lactic acid bacteria was studied by Peterson, Pruess, and Fred (1928). The organisms studied were grown in a medium of glucose and yeast water, or in glucose, yeast water with either peptone or sodium caseinate added. The acid formed by the fermentation of the glucose was neutralized by the presence of calcium carbonate. As an indication of the extent of proteolysis, an analysis of the culture for non-protein nitrogen, amino nitrogen, and ammonia nitrogen was made. It was found that non-protein nitrogen was the most abundant form of nitrogen in the culture medium. The maximum increase of non-protein nitrogen occurred in media containing sodium caseinate, while only a very small amount of ammonia was produced. It was also found that proteolysis continued after all of the sugar had been utilized. Tarnanen (1930), presented data on the proteolytic activity of Bacterium casei epsilon. He reported that the cells of this bacterium which were killed with toluol split casein, gelatin and peptone at an optimum pH of 6, with a range of pH 4 to 8. The optimum temperature for these reactions was stated to have been 42 C. Upon autolysis of the bacterial mass with 40 per cent glycerol, an extract was obtained which hydrolysed casein and peptone. A dipeptidase was present in the crude glycerol extract of the cells, since the cell-free solution split glycylalanine. A purified preparation of a polypeptidase was obtained by adsorption on aluminum hydroxide. This material had an optimum at pH 7.

Frazier and Rupp (1931), studied the action of proteolytic cocci and proteolytic bacilli on milk serum. They showed that most of the

organisms which were capable of breaking down casein also attacked the lactalbumin present in the milk serum, and also reported that Lactobacillus casei was able to break down casein as well as lactalbumin. The distribution of nitrogen compounds resulting from the proteolytic action of certain lactic acid streptococci on milk proteins was studied by Kelly (1931, 1932). The nitrogen changes in milk with added calcium carbonate and the nitrogen changes in milk to which no calcium carbonate had been added were found to progress in a uniform manner, but the proteolysis was found to be greater where the calcium carbonate was present and the cultures were frequently agitated. Gorini, Grassmann and Schleich (1932), studied a group of cheese ripening organisms which in addition to producing lactic acid from lactose also produced a filterable protease. The proteolytic enzyme obtained was a proteinase which digested gelatin at an optimum pH 7.0, and digested the casein of milk at an optimum pH 6.0. Peptidases were not found in the filtrate although they were present in dried preparations of the bacterial cells. Amundstad (1950), investigated the proteolytic activity of rennin and cell-free juice of some lactic acid bacteria associated with the ripening of hard rennet curd cheeses. The organisms used for the preparation of cell-free extracts included Streptococcus lactis, Streptococcus cremoris, Streptococcus diacetilactis and Streptobacterium casei. The enzyme extract was prepared by treatment of the dry cell mass with 87 per cent glycerol at 20 C for 3 days. The proteolytic activity of the bacterial extracts so obtained was studied using casein and peptone solutions as substrates at various pH values.

In general, this author reported that rennin showed considerable proteolytic activity against caseinate solution at pH 5.1 after 9 days of incubation. The pH optima for the endoenzymes of the bacterial cells ranged within the values 6.0 to 7.0. It was also reported that the proteolytic activity of the rennin, as well as the bacterial enzymes, was adversely affected when a sodium chloride concentration of over 5 per cent was present in the reaction mixture.

Although lactic acid bacteria have been shown to possess proteolytic enzymes, as is indicated by the preceeding review of the pertinent literature, a search of the papers dealing with bacterial endoenzymes published since 1932 revealed only one, Amundstadt (1950), which dealt with cell-free proteolytic endoenzymes of lactic acid bacteria. However, the work presented in this paper was very much limited since the extracts obtained were tested only against casein and peptone as substrates. The objects of the present work therefore were:

1. To determine the relative proteolytic activity of various strains of Lactobacillus casei.
2. To find a convenient and reliable method for obtaining crude cell-free extracts from an actively proteolytic strain of Lactobacillus casei;
3. To study the proteolytic activity of the cell-free extracts when these are tested against several types of proteins, peptones, and chemically defined peptides with respect to pH, and the influence of various chemicals as activators or inhibitors.

II

EXPERIMENTAL METHODS

A. Cultures

A collection of fourteen strains of Lactobacillus casei (Orla-Jensen) Holland, was obtained from the Bureau of Dairy Industry, United States Department of Agriculture, Washington, D. C., through the kindness of Dr. Ralph P. Tittsler. These organisms were grown in litmus milk, fresh skim milk, and for morphological studies were cultured in trypticase-yeast extract-glucose broth. Table 1 shows the designation and history of each of the strains.

Gram-stained smears of the organisms were prepared from broth cultures grown at 37 C for 24 hours. Microscopic examination of these preparations revealed the presence of gram-positive, non-sporeforming rods of variable length and width. Some of the strains studied showed the presence of darkly staining granules.

Stock cultures of the organisms were maintained in litmus milk and in agar stabs of the following composition: trypticase, 1.0 per cent; yeast extract 0.5 per cent; glucose, 0.5 per cent; dipotassium hydrogen phosphate, 0.5 per cent; agar, 2.0 per cent; final pH of medium 7.3. The organisms were grown in the above medium for 48 hours and were then kept in the refrigerator. Transfers were made every two months to insure continued viability of the strains. This was accomplished by transferring from a litmus milk culture to a tube of trypticase-yeast extract-glucose broth of the following composition:

Table 1

Number and history of fourteen strains of
Lactobacillus casei

USDA No.	History and Source of Cultures
V300	ATCC 7469; Strong, Wisconsin riboflavin assay
V302	ATCC 334; Sherman, Emmental cheese
V303	ATCC 393; Orla-Jensen strain 7
V311	ATCC 9595; Merck LD 5
V318	ATCC 4943; Orla-Jensen *plantarum* 33; Tittsler considers it to be *casei*
V322	Pederson L 29
V331	ATCC 8041; Snell, *Lactobacillus pentosus*
V339	California Department of Agriculture A 13
V708	Prucha A 5; may have been Kulp 4B
V713	Rosebury 63; Kulp, caries culture
V726	Pederson L 37; E. Kral, *Bacterium casei*
V733	Pederson L 52; Davis, *Streptobacterium casei* 2-11
V743	Pederson F 17-5; Fred, *Lactobacillus arabinosus*
V763	Pederson L 13; Orla-Jensen, *Streptobacterium casei* 7

trypticase, 1.0 per cent; yeast extract, 0.5 per cent; glucose, 1.0 per cent; dipotassium hydrogen phosphate, 0.5 per cent; final pH 7.3. These cultures were incubated at 37 C for 48 hours. At the end of this time, stab inoculations were made into the trypticase-yeast extract-glucose agar already described. These tubes were incubated suitably, and then kept in the refrigerator at 6 to 10 C.

B. Determination of proteolytic activity of ten strains of Lactobacillus casei

Fresh skim milk was distributed in 250 ml quantities in 500 ml flasks, to each of which was added 8 gm of reagent grade powdered calcium carbonate. These were sterilized in the autoclave for 20 minutes at 121 C and were then cooled immediately in the refrigerator. Before use, the flasks were tested for sterility by incubation at 37 C for 48 hours.

The flasks were inoculated with 5 ml of a 48 hour old skim milk culture of each of the organisms tested. After thoroughly shaking each flask in order to insure even distribution of inoculum and calcium carbonate, the pH was determined using the glass electrode of the Beckman pH meter. The flasks were incubated at 37 C for twelve weeks. During the period of incubation, the flasks were shaken daily in order to insure neutralization of the lactic acid formed during growth of the organisms. After incubation the flasks were weighed, and each flask was brought to its initial weight by adding a suitable quantity of sterile distilled water. Determination of the viable Lactobacillus casei cell count, and sterility tests were performed using trypticase-yeast extract-glucose agar as well as nutrient agar. These data are summarized in Tables 2 and 3.

Table 2

Composition of calcium carbonate-skim milk cultures used to determine casein hydrolysis

Flask No.	U.S. Dept. Agr. No.	Initial pH of culture	pH after incubation	Initial weight of flask	Final weight of flask
1	V 302	6.74	6.91	416 gm	382 gm
2	V 303	6.74	6.91	419 "	393 "
3	V 311	6.74	6.93	425 "	396 "
4	V 339	6.74	6.80	408 "	392 "
5	V 318	6.74	7.01	425 "	412 "
6	V 708	6.74	6.77	410 "	393 "
7	V 713	6.70	6.90	413 "	386 "
8	V 726	6.71	6.93	412 "	401 "
9	V 733	6.74	6.80	412 "	383 "
10	V 322	6.70	6.91	426 "	415 "
Plain milk		6.40	6.39	389 gm	376 gm
Calcium carbonate skim milk medium		6.74	6.73	415 "	398 "

Table 3

Plate counts of calcium carbonate-skim milk cultures of *Lactobacillus casei*

Flask No.	Organism No.	Colony count per ml on trypticase-glucose-yeast extract agar	Colony count per ml on nutrient agar
1	V 302	700×10^6	0
2	V 303	35×10^6	0
3	V 311	261×10^6	0
4	V 339	320×10^6	0
5	V 318	500×10^6	0
6	V 708	400×10^6	0
7	V 713	10×10^6	0
8	V 726	300×10^6	0
9	V 733	80×10^6	0
10	V 322	60×10^6	0
Skim milk		0	0
Calcium carbonate skim milk medium		0	0

C. Determination of soluble nitrogen in cultures

The cultures were shaken thoroughly after the addition of sterile distilled water in sufficient quantity to attain the original weight of the flask and contents. About 20 ml of the contents of each flask were aseptically removed and centrifuged for 20 minutes at 4000 rpm. The straw colored supernatant liquid was carefully poured off the residue in the centrifuge tubes. In order to remove protein from the culture centrifugate, the following modification of the method given by Seibert (1926) and Sanders (1933) was employed. Eight ml of 10 per cent trichloracetic acid was added to 2 ml of culture centrifugate. This mixture was allowed to stand for 5 minutes at room temperature, and was then centrifuged for 20 minutes at 4000 rpm in order to remove the precipitated protein. The clear supernatant liquid was poured off the residue and represented a solution of nitrogen compounds not precipitated by trichloracetic acid. To 1 ml of the solution obtained as given above was added 1 ml of concentrated sulfuric acid and a small amount of copper sulfate - potassium sulfate mixture. This material was then digested using a semimicro - Kjeldahl digestion apparatus. The ammonia liberated was determined by titration using 0.0127N sulfuric acid. Each ml of this acid is equivalent to 0.1779 mg of nitrogen. Total nitrogen determinations were performed on the calcium carbonate-skim milk and plain milk controls.

D. Preparation of mass cultures of *Lactobacillus casei* 318

In order to obtain large amounts of cells for the extraction of the endoenzymes, broth of the following composition was used: trypticase, 1.0 per cent; yeast extract, 1.0 per cent; glucose, 0.5 per cent; ammonium sulfate, 0.5 per cent; dipotassium hydrogen phosphate, 0.5 per cent; magnesium sulfate, 0.01 per cent; manganous sulfate, 0.001 per cent; final pH 7.3.

Five hundred ml quantities of this broth were distributed in one liter flasks. After autoclaving, the flasks were cooled rapidly in order to reduce caramelization, and then were incubated for 48 hours at 37 C. Each sterile flask was then inoculated with 5 ml of a 24 hour trypticase-yeast extract-glucose broth culture of *Lactobacillus casei* 318. These inoculated flasks were incubated for 48 hours at 37 C. The organisms grew rapidly in this broth and the cells were collected by centrifugation in a Sharples Supercentrifuge. The cells were separated as a thick, greyish white, moist paste, on the inner walls of the bowl of the centrifuge. The resulting cell paste was suspended in sufficient distilled water to yield a suspension containing approximately 1 gm wet weight of cells per ml.

E. Methods used to prepare crude cell-free extracts

1. Grinding cells with sand

Five ml of suspension containing 4.5 to 5.0 gm wet weight of cells, were mixed with 10 gm of washed and ignited quartz sand in a

porcelain mortar. The cell-sand mixture was ground at room temperature by hand for 2 minutes. As grinding proceeded, any of the cell-sand mixture which collected at the sides of the mortar was scraped away, so that it came into contact with the grinding surface of the pestle. The cell-sand mass had the consistency of thick plaster of paris. Ten ml of water was added to the mortar and the ground cell mass was transferred to a centrifuge tube. This was centrifuged at 2000 rpm for 30 minutes. The supernatant liquid was then decanted and recentrifuged.

2. Freezing and thawing

Fifteen ml of suspension of Lactobacillus casei 318 were placed in a 50 ml flask. To this was added 10 ml of distilled water. The suspension was placed in the freezing compartment of the refrigerator. Long crystals of ice were seen to form within 20 minutes, and the entire mass was solidly frozen within one hour. This mass was allowed to thaw at room temperature and the process was repeated four times on each of six successive days. The crude enzyme extracts were separated from debris by centrifugation at 2000 rpm for 30 minutes.

3. Grinding with powdered alumina

To 10 gm weight of cell paste was added 20 gm of Fisher Co. Alumina 80/100 mesh. The alumina was mixed with the cell paste in a centrifuge tube. The resulting damp paste was transferred to a mortar and the mixture was ground by hand for five minutes. Ten ml of distilled water were now added and the grinding was continued for 2 minutes. An additional 20 ml of distilled water was added and the suspension was transferred to

a centrifuge tube. After centrifugation at 2000 rpm for 30 minutes, the crude extract was decanted from the sediment.

4. Grinding with alumina and extracting mass with M/15 disodium phosphate

Twenty gm of Fisher alumina 80/100 mesh were added to 10 gm wet weight of packed cells. This mixture was ground according to the procedure outlined previously. The ground suspension was centrifuged at 2000 rpm for 30 minutes. The slightly turbid, greyish white supernatant liquid of pH 6.7 which was obtained was called extract A and was stored in the refrigerator until used. To the residue remaining in the centrifuge tubes was added 10 ml of M/15 disodium phosphate solution at pH 9.1. The alumina-cell residue was thoroughly stirred in the phosphate solution for five minutes. This was then centrifuged and the resulting supernatant fluid was decanted. The extraction of the residue was repeated as given above. The combined phosphate solutions were called extract B and were stored in the refrigerator until used.

5. Grinding with Alcoa E-20 (alumina)

Ten gm of Alcoa E-20 alumina were added to about 5 gm wet weight of packed cells of Lactobacillus casei 318. A stiff, somewhat dry paste was obtained which was transferred to a porcelain mortar and ground by hand for 5 minutes. To this paste was added 5 ml of distilled water and the grinding was continued for an additional 5 minutes. Finally, 15 ml of distilled water were added and the resulting suspension was centrifuged at 4000 rpm for 30 minutes. A slightly turbid, tan supernatant liquid was obtained which had a pH of 7.3. The original alumina-cell suspension had a pH of 8.2. The proteolytic activity of the extract

obtained as given above was determined using casein as substrate buffered and unbuffered at pH 8.9. The proteolytic activity of a commercial trypsin preparation was determined under similar conditions of pH and temperature.

6. Extraction with 40 per cent glycerol

To 3 gm wet weight of packed cells of Lactobacillus casei 318, was added 20 ml of 40 per cent glycerol. The cells were thoroughly stirred in the glycerol solution for 5 minutes. At the end of that time, 0.5 ml of toluene was added and the mixture was allowed to stand at room temperature for 24 hours. The mixture was then centrifuged at 4000 rpm for 30 minutes, and the 40 per cent glycerol extract was separated from the packed debris and toluene.

The preparation of a glycerol extract as described above was repeated using 5 gm wet weight of packed cells and 35 ml of 40 per cent glycerol solution. After centrifugation of the mixture, a very pale yellow, slightly turbid solution was obtained.

7. Shaking cell suspension with glass beads 5 mm in diameter

Glass beads 5 mm in diameter were cleaned in aqua regia, washed in distilled water and dried in the oven at 160 C for 3 hours. Thirty grams of these beads were placed in a six ounce screw capped prescription bottle. Fifteen ml (containing about 4.3 gm wet weight of cells) of a suspension of Lactobacillus casei 318 were added followed by 5 ml of 1M ammonia-ammonium chloride buffer pH 8.9 and 10 ml of distilled water. The bottle was affixed to an International Shaking Machine and

it was shaken at the highest speed of the machine. After two hours of shaking 10 ml of the suspension were removed and the resulting suspension was shaken an additional two hours. Cell-free crude extracts were prepared by centrifuging 10 ml of the shaken suspensions at 4000 rpm for 20 minutes. A clear, light yellow solution was obtained which was at pH 8.2. The proteolytic activity of both the 2 hour and 4 hour extract was tested using casein buffered at pH 8.9 and dl-leucylglycine unbuffered at pH 6.3.

Fifteen ml (containing about 4.3 gm wet weight of cells) were shaken with 30 gm of glass beads, 10 ml of M/15 phosphate buffer pH 7.0, and 5 ml of distilled water. Another suspension was prepared as described above except that 10 ml of M/15 phosphate buffer pH 6.6 was substituted for the one at pH 7.0. These buffered cell suspensions were shaken for two hours at the maximum speed of the International Shaking Machine. After centrifuging, the proteolytic activity of the extracts was tested using casein as substrate buffered at pH 7.0 and 6.6. Similar suspensions were prepared as above, but the pH was adjusted using 1M sodium acetate-acetic acid buffer pH 5.8 and 1M ammonia-ammonium chloride buffer at pH 8.9. The proteolytic activities of these extracts were determined using casein, trypticase, trytone, proteose-peptone and tryptose.

8. Shaking with pavement marking beads at pH 7.0

Twenty-five gm of very fine glass pavement marking beads 60/80 mesh, were placed in a clean six ounce screw-capped prescription bottle.

Twenty ml of Lactobacillus casei 318 containing about 6 gm wet weight of cells were added followed by 10 ml of M/15 phosphate buffer at pH 7.0, and 10 ml of distilled water. Shaking was carried out as given above for 2 and 4 hours. The proteolytic activity of this extract was determined using casein as substrate buffered at pH 8.9.

9. Shaking cells in high-intensity ultrasonic generator

The disruption of bacterial cells by irradiation with ultrasonic vibrations has been considered as a very suitable method by which the preparation of cell constituents could be accomplished. Thus, Chambers and Flosdorf (1936), and Stumpf, Green, and Smith (1946), used the ultrasonic disintegration method as a means of obtaining labile bacterial cell constituents. Attempts to produce a cell-free active extract from Lactobacillus casei 318 by ultrasonic irradiation, was carried out using a piezoelectric high-intensity ultrasonic generator of the type described by Campbell and Shoenleber (1949). The method used for treatment of the cells was carried out as described below.

Ten ml suspension of the cells were mixed with 2 ml of acetate buffer at pH 5.8 and 13 ml of distilled water. One ml of this suspension was diluted with 20 ml of distilled water. The resulting suspension was placed in a large pyrex test tube which was immersed in a copper cup filled with water. The bacterial suspension was exposed to ultrasonic radiations for periods of 1, 2, 4, 8, and 15 minutes at 400 kilocycles. After each time interval a 4 ml sample was removed for microscopic examination and preparation of crude cell-free extracts

by centrifugation. The extent of disruption of the cells was observed by microscopic examination of thin smears stained by polychrome methylene blue. The proteolytic activity of the extracts was determined by using casein as substrate buffered at pH 5.8.

10. Preparation of extracts by autolysis

(a) The pH of 20 ml of cell suspension containing 3 gm wet weight of cells was adjusted by means of 0.1N sodium hydroxide to 7.3. Two ml of toluene were added, and the mixture was incubated at 37 C for two weeks. After centrifugation a light yellow turbid solution was obtained which had pH of 6.0. The proteolytic activity of this extract was determined using casein, gelatin and trypticase buffered at pH 5.8.

(b) Ten ml of cell suspension were mixed with 10 ml of 2 per cent Tween 80*, and 5 ml of 1M sodium acetate-acetic acid buffer solution at pH 4.5. Two ml of toluene were added and the mixture was incubated at 37 C for two weeks. The proteolytic activity of the cell-free extract obtained after centrifugation was tested using casein, gelatin, and trypticase as substrates, buffered at pH 5.8.

(c) Ten ml of bacterial suspension was mixed with 10 ml of 2 per cent Tween 80 and 6 ml of 1M ammonia-ammonium chloride buffer at pH 8.9. Two ml of toluene were added and the mixture was incubated at 37 C for

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* Polyoxyethylene sorbitan monooleate, made by Atlas Powder Company, Wilmington, Delaware.

two weeks. The proteolytic activity of the cell-free extract obtained was determined as given above except that the substrates were buffered at pH 8.9.

11. Preparation of extract using 1M glycine

A modification of the method prpposed by Cowles (1947) for lysing bacterial cells was performed as follows: About 1 gm wet weight of cells was suspended in sufficient distilled water to give a final volume of 9 ml. To this suspension were added 1 ml of 1M ammonia-ammonium chloride buffer at pH 8.9 and 10 ml of 2M glycine solution. Two ml of toluene were now added and the mixture allowed to incubate at 30 C for 24 hours. A similar preparation of cells and glycine was prepared except that the toluene was omitted and the mixture was incubated at 6 C for 24 hours.

12. Preparation of extract using rennin

Ten ml of a suspension of cells was mixed with 10 ml of 1:100 solution of rennin prepared in acetate buffer at pH 5.0. A second flask was prepared by mixing 10 ml of 1:100 solution of rennin at pH 5.0 with 10 ml of distilled water. A third flask was prepared by mixing 10 ml of cell suspension with 10 ml of 0.1M acetate buffer at pH 5.0. To each of the above mixtures was added 1 ml of toluene, and they were allowed to incubate at 40 C for 48 hours. Cell-free extracts from each of the mixtures were obtained by centrifugation. The milk-clotting power of the extracts prepared as given above was determined according to a modification of the method proposed by Kunitz (1935) and Herriott (1938).

13. Preparation of extract by grinding cells with solid carbon dioxide

Ten gm of solid carbon dioxide were placed in a porcelain mortar which was immersed in a freezing mixture of solid carbon dioxide and alcohol. The carbon dioxide was pulverized and 5 ml of a suspension of cells (containing 1.5 gm wet weight) were added dropwise. This was allowed to stand for 10 minutes, and then the frozen cells were ground by hand with the pulverized carbon dioxide. After grinding for 5 minutes, 20 ml of M/15 phosphate buffer pH 7.0 were added and the grinding was continued for an additional 5 minutes. The greyish-white powder which resulted was placed in the refrigerator at 6 C, and after standing overnight a turbid, grey liquid was obtained. From this solution a cell-free extract was obtained by centrifugation.

14. Preparation of cell-free extracts by the use of the Mickle Tissue Disintegrator

The disintegration of bacterial cells by shaking with hard inert particles was demonstrated by Curran and Evans (1942). These workers found that the greatest amount of disruption occurred when the cells were shaken in the presence of very fine glass beads (sieve numbers of 60/80). Curran and Evans further suggested that more efficient cell disintegration could be obtained by modifying the construction of the shaking apparatus. They indicated that increased amplitude of stroke, increased speed, and refinement of receptacle design to eliminate pockets, crevices and other irregularities might aid in attaining this end. These features are incorporated in the machine described by Mickle

(1948), and which was used in preparing most of the extracts studied.

The disintegration of the lactobacilli used in these studies was accomplished by shaking a suspension in the presence of very fine pavement marking beads (sieve numbers 60/80), in special vials attached to the ends of tuned reeds. These steel reeds are vibrated magnetically by means of a system of electromagnets. The instrument used for this work is shown in fig. 1. The extracts were prepared by pipetting 5 ml of cell suspension containing 1 gm wet weight of cells into a vial containing 8 gm of pavement marking beads.[1] Distilled water was then added to within one-half inch of the top of the vial. Shaking was allowed to proceed for 20 minutes at the maximum amplitude of the vibrating reeds. When necessary the temperature of the contents of the vials was controlled by playing a stream of compressed carbon dioxide as released from a tank against them during the shaking period. After shaking, the suspensions were centrifuged for 30 minutes at 4000 rpm, and a slightly cloudy, light yellow, cell-free extract resulted.

In order to demonstrate the progress of disintegration by the shaking process, microscopic observations of stained smears of the cell suspensions both before and after shaking were made. Electronographs of the cell preparations, both shaken and unshaken, were also made.

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[1] Type S.C.D. 2522-511, made by the Minnesota Mining and Manufacturing Co., Saint Paul, Minnesota.

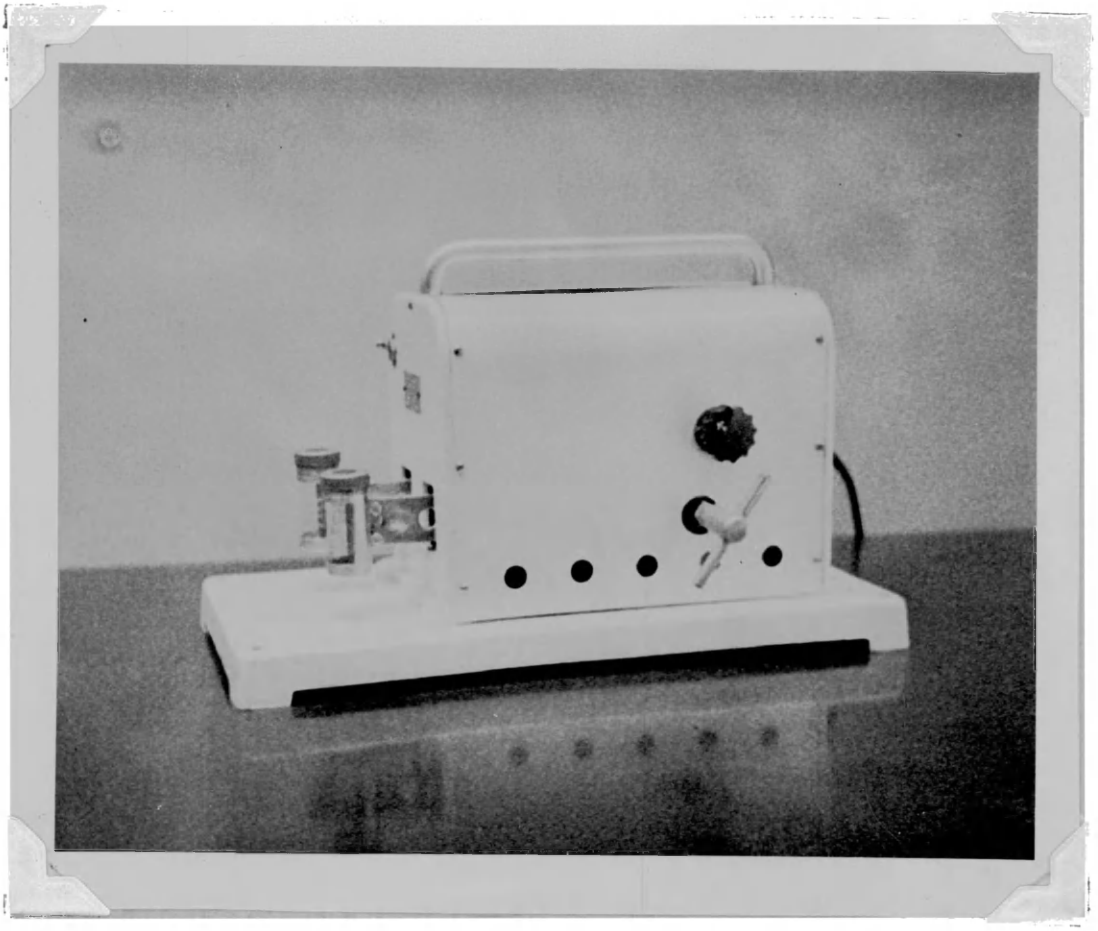

Fig. 1 Mickle Tissue Disintegrator

F. Analytical methods used to determine extent of proteolysis

In order to determine tryptic activity of some of the cell-free extracts, the following modification of the method described by Willstätter, Waldschmidt - Leitz, Dunaiturria, and Kunster (1926) was used.

One ml of the extract prepared by grinding the cells with sand was added to 2 ml of water. This mixture was heated at 30 C for 30 minutes, after which time, 5 ml of 6 per cent casein solution and 2 ml of 1N ammonia-ammonium chloride buffer solution diluted 1:1 were added. The pH of the mixture was thus adjusted to 8.9. This was then incubated at 40 C for 1 hour. A similar mixture was prepared as given above, but the preliminary activation of the extract by heat for 30 minutes at 30 C was omitted. Finally, for each determination a control tube was prepared according to the method given above, except that the extract was boiled for 5 minutes and then filtered before being added to the buffered substrates. The extent of hydrolysis of the casein was determined by titration of the test mixtures after incubation for 1 hour at 40 C. The titration was carried out in 90 per cent alcohol using thymolphthalein as indicator and 0.2N alcoholic potassium hydroxide.

In order to study the protease activity of most of the extracts used in this work, the following general methods were employed. For the detection of proteinases 5 per cent gelatin and 6 per cent casein solutions were used. The casein substrate was prepared by suspending 6 gm of Bacto iso-electric casein in 100 ml of distilled water. Two

ml of 1N ammonia solution were then added dropwise with constant shaking until complete dissolution of the casein was effected.

To detect the presence of polypeptidases, 5 per cent freshly prepared solutions of Bacto peptone, tryptone, trypticase[1], and tryptose were used.

For the detection of the various peptidases chemically defined peptide substrates were used. These were the following: M/15 dl-leucylglycylglycine; M/15 dl-leucylglycine; M/15 dl-alanylglycine; M/15 chloracetyltyrosine; M/30 l-leucylglycine; M/30 glycyl-l-leucine; and M/30 d- leucyl-l-tyrosine.

The proteolytic activity of a particular crude enzyme extract was determined on a mixture of extract, substrate, buffer, and distilled water after a suitable time of incubation at 40 C. The buffer solutions used were the following: 1M acetic acid-sodium acetate, range pH 3.1 to 6.2; McIlwain's buffer solutions, range pH 6.0 to 8.0; 1M ammonia-ammonium chloride, range pH 8.0 to 11.0.

The hydrolysis of the substrates used in both the test and control mixtures was followed by using the following modification of the Willstätter and Waldschmidt-Leitz titration for free carboxyl groups.

Two ml aliquots of the test mixtures after incubation at 40 C for 0,1, and 24 hour periods were pipetted into 20 ml of 90 per cent

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1 Prepared by Baltimore Biological Company, Baltimore, Maryland.

ethanol. One ml of 0.02 per cent thymolphthalein indicator in 90 per cent ethanol was then added and the mixture was titrated with standard alcoholic solution of potassium hydroxide until the appearance of the first blue color. Fifteen ml of boiling 95 per cent ethanol were now added, and the original blue color disappeared. The addition of the standard base was continued until the blue color reappeared. An increase in the titration figure after incubation at 40 C was used as the indication of hydrolysis for a particular substrate used. All the titrations were carried out by using a 10 ml microburete graduated in 0.02 ml units. The volumes delivered by this burette, could be estimated to 0.01 ml.

III

RESULTS

A. Proteolytic activity of several strains of *Lactobacillus casei*

In the initial investigations of the proteolytic activity of *Lactobacillus casei*, the ability of ten strains of this organism to decompose the casein in calcium carbonate-skim milk medium was studied. Table 4 shows the results obtained when the content of the trichloracetic acid soluble nitrogen compounds present in the cultures was determined. Although the results give no indication of the extent of breakdown of the casein, they do show however, that all of the strains tested possessed a proteinase capable of degrading the casein. The degree of activity of the strains varied. Since strain 318 showed the greatest activity, it was selected as the source from which cell-free crude extracts were to be prepared for further study.

B. Mass culture of *Lactobacillus casei* 318

Since, in any study of bacterial endoenzymes, large amounts of cell substance is necessary, it was important at the outset of this work to obtain a medium which would yield good quantities of cells per unit volume of medium. In addition, since large quantities of medium would be required it was important from the practical standpoint to use a medium both relatively simple in composition as well as one which was inexpensive. Among the several media which were tried during a few preliminary trials, the most satisfactory one was the broth already described under Experimental Methods. This broth gave excellent yields of all strains

Table 4

Nitrogen determinations of 12 week cultures of ten strains of *Lactobacillus casei*

U.S. Dept. Agr. No.	ml 0.0127N acid used per 0.2 ml aliquot filtrate	mg soluble nitrogen per ml filtrate	soluble nitrogen formed, in per cent of total nitrogen
V.302	1.50	1.33	17.5
V 303	2.00	1.78	25.9
V 311	1.71	1.52	20.8
V 318	2.59	2.31	35.7
V 322	0.53	0.47	1.3
V 339	1.98	1.76	25.5
V 708	1.50	1.33	17.5
V 713	2.30	2.05	30.9
V 726	1.71	1.52	21.0
V 733	2.05	1.78	25.9
$CaCO_3$-skim milk control	0.45	0.40	

Total nitrogen = 5.33 mg per ml

of Lactobacillus casei. An average of 25 to 30 gm wet weight of packed cells was obtained upon centrifugation of 20 liters of culture after 48 hours of incubation at 37 C.

C. Relative effectiveness of methods used to prepare cell-free extracts

Before studies of the various aspects of the proteolytic activity of crude cell-free extracts could commence, it was important to find the most efficient and reliable means of preparation of such an extract. In order to achieve this end various methods were used to disrupt the bacterial cells and to obtain the cell contents for the determination of the presence of proteolytic enzymes. The cells of Lactobacillus casei were found to be very resistant to various physical and chemical agents. They were very resistant to changes in salt concentration, with the concomitant changes in osmotic pressure, and were also resistant to changes in pH.

1. Grinding cells with sand

A microscopic examination of the cell suspension obtained after grinding them with sand according to the procedure already outlined revealed only a very small degree of cell desintegration. The proteolytic activity of the cell-free extract when tested against casein is shown by the titration figures recorded in table 5. The increase in acidity of the test mixtures over the control indicates that the crude extract possessed a proteinase capable of causing some degradation of the casein. However, these extracts were not satisfactory since preparations of high

Table 5

Tryptic activity of extract from sand ground cells

Tube No.	ml 0.2N alcoholic KOH used per 10 ml
1	6.33
2	6.33
control	6.18

Table 6

Tryptic activity of extract prepared by alternate freezing and thawing

Tube No.	ml 0.2N alcoholic KOH used per 10 ml
1	2.27
2	2.31
control	2.32

and uniform activity could not be obtained.

2. Freezing and thawing

The microscopic examination of the suspension obtained after the freezing and thawing process showed no change in cell morphology thus indicating no disruption of the cell with a liberation of its contents. When the proteolytic activity of this extract was tested against casein as substrate, the titration figures as shown in table 6 reveal no proteolytic activity. Although Berger, Johnson and Peterson (1938a), reported the successful preparation of bacterial endoenzymes by this method, it was apparently not useful for the disintegration of cells of Lactobacillus casei.

3. Grinding with powdered alumina

In view of the fact that a proteolytically active extract could be procured by a grinding procedure, it was decided to study other abrasives for the production of a cell-free extract. McIlwain (1948), suggested the use of powdered alumina as a medium useful for aiding the disintegration of bacterial cells when they were ground with it. This author reports that it was possible to break over 99 per cent of streptococcal cells by this procedure, but microscopic examination of gram stained preparations of Lactobacillus casei 318 after grinding with alumina revealed only very few destroyed cells. The proteolytic activity of the extracts prepared in this manner were tested against trypticase, casein, and gelatin as substrates. The titration figures shown in table 7 indicate the absence in the extract of a polypeptidase acting on trypticase.

Table 7

Polypeptidase activity of extract
obtained by grinding cells with alumina

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml trypticase+2 ml extract 1 ml buffer pH8.9+5 ml water	2.32	2.32	2.32	0.00
2	2 ml trypticase+2 ml extr.boiled 1 ml buffer pH8.9+5 ml water	2.30	2.31	2.31	0.00
3	2 ml trypticase+1 ml buffer pH 8.9 7 ml water	2.50	2.50	2.50	0.00
4	2 ml trypticase+ 2 ml extract 1 ml buffer pH 6.0 + 5 ml water,	0.30	0.30	0.30	0.00
5	2 ml trypticase + 2 ml extr.boiled 1 ml buffer pH 6.0 + 5 ml water	0.29	0.28	0.28	0.00
6	2 ml trypticase + 1 ml buffer pH 6.0+7 ml water	0.30	0.30	0.30	0.00

Tables 8 and 9 show the values obtained when the extract was tested against casein and gelatin. The results indicate the absence of a proteinase in the extract.

4. Grinding with alumina and extracting mass with M/15 disodium phosphate solution

In order to determine whether the alumina had adsorbed any proteinase which may have been liberated from the cells, the ground cell mass was extracted with water (extract A), followed by another extraction with M/15 disodium phosphate solution (extract B). Table 10 shows the results obtained using extract A tested against gelatin, casein, and trypticase. No proteolytic activity of this extract is indicated. However, the titration figures recorded in table 11 show that extract B contained both a proteinase activity against casein and gelatin, as well as a polypeptidase which was capable of hydrolysing trypticase. Although activity against all three substrates can be noted, the greatest activity was elicited when casein was used as a substrate.

5. Grinding with Alcoa E-20 alumina

In further attempts to improve the efficiency of the grinding of the lactobacilli cells, thereby obtaining a more active extract, a specially prepared alumina (Alcoa E-20) was tried. It was thought at this point, that the apparent inactivity of the extracts which were tested might not be due to lack of enzyme content, but in reality, might be attributed to some defect in the substrate itself. Therefore, in order to eliminate this objection, a positive control consisting of a suitable trypsin preparation was included in the tests. The results of

Table 8

Caseolytic activity of extract obtained by grinding cells with alumina.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml extract 1 ml buffer pH 8.9 + 5 ml water	2.64	2.65	2.64	0.00
2	2 ml casein + 2 ml extr.boiled 1 ml buffer pH 8.9 + 5 ml water	2.60	2.60	2.60	0.00
3	2 ml casein + 1 ml buffer pH 8.9 7 ml water	2.55	2.56	2.56	0.01
4	2 ml casein + 2 ml extract 1 ml buffer pH 6.0 + 5 ml water	0.50	0.50	0.50	0.00
5	2 ml casein + 2 ml extr. boiled 1 ml buffer pH 6.0 + 5 ml water	0.47	0.47	0.47	0.00
6	2 ml casein + 1 ml buffer pH 6.0 7 ml water	0.45	0.45	0.45	0.00

Table 9

Gelatinolytic activity of extract obtained by grinding cells with alumina.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml gelatin + 2 ml extract 1 ml buffer pH 8.9 + 5 ml water	2.45	2.44	2.44	0.00
2	2 ml gelatin + 2 ml extr.boiled 1 ml buffer pH 8.9 + 5 ml water	2.40	2.42	2.40	0.00
3	2 ml gelatin + 1 ml buffer pH 8.9 7 ml water	2.16	2.16	2.16	0.00
4	2 ml gelatin + 2 ml extract 1 ml buffer pH 6.0 + 5 ml water	0.28	0.27	0.27	0.00
5	2 ml gelatin + 2 ml extr. boiled 1 ml buffer pH 6.0 + 5 ml water	0.25	0.25	0.25	0.00
6	2 ml gelatin + 1 ml buffer pH 6.0 7 ml water	0.22	0.23	0.22	0.00

Table 10

Proteolytic activity of extract obtained by extracting alumina-cell mass with water.

Tube No.	Test Mixture	ml KOH used Hour 0	1	24	Increase in acidity
1	2 ml casein + 2 ml extract A 1 ml buffer pH 8.9 + 5 ml water	1.93	1.91	1.93	0.00
2	2 ml casein + 2 ml extr. A boiled 1 ml buffer pH 8.9 + 5 ml water	1.93	1.92	1.90	0.00
3	2 ml gelatin + 2 ml extract A 1 ml buffer pH 8.9 + 5 ml water	1.68	1.69	1.68	0.00
4	2 ml gelatin + 2 ml extr. A boiled 1 ml buffer pH 8.9 + 5 ml water	1.67	1.68	1.66	0.00
5	2 ml trypticase + 2 ml extr. A 1 ml buffer pH 8.9 + 5 ml water	1.74	1.74	1.75	0.01
6	2 ml trypticase + 2 ml extr.A boiled 1 ml buffer pH 8.9 + 5 ml water	1.76	1.75	1.75	0.00

Table 11

Proteolytic activity of extract obtained by extracting alumina-cell mass with M/15 phosphate solution.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml extract B 1 ml buffer pH 8.9 + 5 ml water	1.93	1.95	2.01	0.08
2	2 ml casein + 2 ml extr. B boiled 1 ml buffer pH 8.9 + 5 ml water	1.90	1.90	1.88	0.00
3	2 ml gelatin + 2 ml extract B 1 ml buffer pH 8.9 + 5 ml water	1.68	1.70	1.74	0.06
4	2 ml gelatin + 2 ml extr. B boiled 1 ml buffer pH 8.9 + 5 ml water	1.68	1.66	1.66	0.00
5	2 ml trypticase + 2 ml extract B 1 ml buffer pH 8.9 + 5 ml water	1.78	1.81	1.84	0.06
6	2 ml trypticase + 2 ml extr. B boiled 1 ml buffer pH 8.9 + 5 ml water	1.76	1.75	1.75	0.00

these titrations are recorded in table 12.

It is apparent that the Alcoa E-20 extract contained no proteolytic enzymes since no increase in the titration figures is noted. However, the trypsin solution caused considerable hydrolysis of the casein substrate.

6. Extraction with 40 per cent glycerol

The use of glycerol solution as a means of extracting enzymes from cells has been in use for many years and has been successful when various species of bacteria and yeasts have been used.

In the work described here, when a cell mass of Lactobacillus casei 318 was treated with 40 per cent glycerol, the extract which was obtained was capable of hydrolysis casein, trypticase, tryptone, proteose-peptone, and tryptose. Table 13 shows the results of the titration when casein and dl-leucylglycine were used as substrates. The figures indicate that the extract contained a proteinase capable of hydrolysing casein, but did not contain a dipeptidase which could attack the dipeptide.

The results of the determination of enzyme activity performed on various peptones are recorded in table 14. Although active extracts could be prepared by the use of glycerol these extracts proved undesirable for several practical reasons. It was found that the comparatively high viscosity of the glycerol solution made accurate pipetting very difficult since the flow of the liquid was not uniform. In addition, the glycerol solution tended to foam. It also interfered with the accurate determination of the end point of the alcohol titration.

Table 12

Proteolytic activity of extract from cells ground with Alcoa E-20.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml extract 1 ml buffer pH 8.9 + 5 ml water	1.85	1.85	1.85	0.00
2	2 ml casein + 2 ml extr. boiled 1 ml buffer pH 8.9 + 5 ml water	1.82	1.83	1.82	0.00
3	2 ml casein + 1 ml buffer pH 8.9 7 ml water	1.84	1.85	1.85	0.01
4	2 ml extract + 1 ml buffer pH 8.9 7 ml water	1.67	1.67	1.67	0.00
5	2 ml casein + 2 ml 1 per cent trypsin 1 ml buffer pH 8.9 + 5 ml water	2.32	2.37	2.44	0.12
6	2 ml 1 per cent trypsin + 7 ml water 1 ml buffer pH 8.9	0.49	0.47	0.49	0.00

Table 13

Proteolytic activity of glycerol extract using casein and dl-leucylglycine as substrates.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml extract 1 ml buffer pH 8.9 + 5 ml water	1.05	1.12	1.14	0.09
2	2 ml casein + 2 ml extr. boiled 1 ml buffer pH 8.9 + 5 ml water	1.05	1.05	1.05	0.00
3	2 ml casein + 1 ml buffer pH 8.9 7 ml water	1.05	1.05	1.05	0.00
4	2 ml extract + 1 ml buffer pH 8.9 7 ml water	0.89	0.89	0.89	0.00
5	2 ml M/15 dl-leucylglycine 2 ml extract + 6 ml water	0.63	0.64	0.63	0.00
6	2 ml M/15 dl-leucylglycine 2 ml extract boiled + 6 ml water	0.60	0.62	0.60	0.00
7	2 ml M/15 dl-leucylglycine 8 ml water	0.15	0.13	0.14	0.00

Table 14

Proteolytic activity of glycerol extract using several types of peptones as substrates.

Tube No.	Test Mixture	ml KOH used Hour 0	Hour 1	Hour 24	Increase in acidity
1	2 ml trypticase + 1 ml extract 1 ml buffer pH 5.8 + 6 ml water	2.19	2.21	2.36	0.17
2	2 ml trypticase + 1 ml extr. boiled 1 ml buffer pH 5.8 + 6 ml water	2.17	2.16	2.16	0.00
3	2 ml tryptone + 1 ml extract 1 ml buffer pH 5.8 + 6 ml water	2.46	2.49	2.65	0.19
4	2 ml tryptone + 1 ml extr. boiled 1 ml buffer pH 5.8 + 6 ml water	2.41	2.42	2.39	0.00
5	2 ml proteose-peptone + 1 ml extr. 1 ml buffer pH 5.8 + 6 ml water	1.82	1.88	2.10	0.28
6	2 ml proteose-peptone + 1 ml extr.boiled 1 ml buffer pH 5.8 + 6 ml water	1.80	1.79	1.77	0.00
7	2 ml tryptose + 1 ml extract 1 ml buffer pH 5.8 + 6 ml water	1.74	1.75	1.79	0.05
8	2 ml tryptose + 1 ml extr. boiled 1 ml buffer pH 5.8 + 6 ml water	1.73	1.70	1.70	0.00

7. Shaking cell suspension with glass beads 5 mm in diameter

Although proteolytic extracts could be obtained by extraction of cells with glycerol, other methods for disrupting the Lactobacillus casei cells were studied. It was felt that more suitable extracts might be prepared if the presence of extraneous materials, such as glycerol could be avoided. Since grinding methods, using different abrasives did not satisfactorily attain this end, the preparation of extracts by shaking the bacterial cells under various conditions was examined.

It was found that after comparatively long periods of shaking in an International Shaking Machine, in the presence of glass beads and suitable buffers, cell-free extracts which were proteolytically active could be prepared. When the cells were shaken in the presence of ammonia buffer at pH 8.9, an active extract was obtained after 2 hours of shaking. The activity, as elicited against casein used as substrate was not diminished or destroyed even when the shaking was continued for a total time of 4 hours. In table 15 are recorded the results of the determinations using both 2 hour and 4 hour extracts, tested against casein buffered at pH 8.9. These extracts contained a caseolytic proteinase, but did not contain enzymes capable of hydrolysing peptones, or the racemic dipeptide, dl-leucylglycine. The results for these determinations are shown in tables 16 and 17.

When extracts were prepared by shaking a cell suspension in the presence of buffers at pH 7.0 and pH 6.6, and then tested against

Table 15

Caseolytic activity of extract obtained by shaking cells with 5 mm glass beads at pH 8.9 for 2 and 4 hour periods.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml 2 hr. extract 1 ml buffer pH 8.9 + 5 ml water	1.87	1.89	1.95	0.08
2	2 ml casein + 2 ml 2 hr. extr. boiled 1 ml buffer pH 8.9 + 5 ml water	1.87	1.86	1.85	0.00
3	2 ml 2 hr. extract + 7 ml water 1 ml buffer pH 8.9	1.09	1.10	1.10	0.01
4	2 ml casein + 2 ml 4 hr. extract 1 ml buffer pH 8.9 + 5 ml water	1.86	1.89	1.98	0.12
5	2 ml casein + 2 ml 4 hr. extr. boiled 1 ml buffer pH 8.9 + 5 ml water	1.82	1.83	1.83	0.01
6	2 ml 4 hr. extract + 7 ml water 1 ml buffer pH 8.9	1.17	1.18	1.18	0.01
7	2 ml casein + 7 ml water 1 ml buffer pH 8.9	0.82	0.84	0.83	0.01

Table 16

Proteolytic activity of extract obtained by shaking cells with 5 mm glass beads at pH 8.9 for 4 hours using peptones as substrates.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml trypticase + 1 ml extract 1 ml buffer pH 8.9 + 6 ml water	1.73	1.75	1.73	0.00
2	2 ml trypticase + 1 ml extr. boiled 1 ml buffer pH 8.9 + 6 ml water	1.51	1.51	1.50	0.00
3	2 ml tryptone + 1 ml extract 1 ml buffer pH 8.9 + 6 ml water	1.63	1.60	1.62	0.00
4	2 ml tryptone + 1 ml extr. boiled 1 ml buffer pH 8.9 + 6 ml water	1.56	1.56	1.56	0.00
5	2 ml proteose-peptone + 1 ml extr. 1 ml buffer pH 8.9 + 6 ml water	1.49	1.47	1.50	0.00
6	2 ml proteose-peptone + 1 ml extract boiled + 1 ml buffer pH 8.9 6 ml water	1.47	1.48	1.48	0.01
7	2 ml tryptose + 1 ml extract 1 ml buffer pH 8.9 + 6 ml water	1.69	1.70	1.70	0.01
8	2 ml tryptose + 1 ml extract boiled 1 ml buffer pH 8.9 + 6 ml water	1.40	1.40	1.40	0.00

Table 17

Dipeptidase activity of extract obtained by shaking cells with 5 mm glass beads for 2 and 4 hours using M/15 dl-leucylglycine as substrate.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml M/15 dl-leucylglycine 2 ml 2 hr. extract + 6 ml water	1.25	1.26	1.25	0.00
2	2 ml M/15 dl-leucylglycine 2 ml 2 hr. extr. boiled + 6 ml water	1.23	1.23	1.23	0.00
3	2 ml M/15 dl-leucylglycine 2 ml 4 hr. extract + 6 ml water	1.40	1.41	1.38	0.00
4	2 ml M/15 dl-leucylglycine 2 ml 4 hr. extr. boiled + 6 ml water	1.35	1.37	1.33	0.00
5	2 ml M/15 dl-leucylglycine 8 ml water	0.14	0.15	0.15	0.01

casein substrate also buffered at pH 7.0 and ph 6.6 respectively, no activity could be detected. The extracts prepared at these pH values also would not hydrolyze any of the peptones used. The titration values obtained for these experiments are recorded in table 18.

When a cell suspension was shaken at the acid pH level of 5.8 the preparation of proteolytically active extracts could be accomplished. It was possible to produce extracts at this pH value which contained a proteinase capable of hydrolysing casein at pH 5.8 as well as a polypeptidase capable of degrading trypticase, tryptone, proteose-peptone, and tryptose. The results of these analyses are recorded in tables 19 and 20. It appears that shaking the cells results in the extraction of a proteinase which acts at both an alkaline and an acid pH level. However, the polypeptidase activity of the extract could be detected only when it was prepared from the cells at pH 5.8 and tested against the various peptone substrates at the same pH value.

8. Shaking with pavement marking beads

The results of the previous experiments just discussed, indicate that, under suitable conditions proteolytically active extracts from the cells of Lactobacillus casei can be obtained by shaking in the presence of rather large size glass beads. Repeated microscopic observations of stained smears of the shaken cell suspensions failed to reveal any extensive cell disintegration, even though active extracts were obtained. In order to increase the chance of cell disintegration, thereby increasing the yield of endoenzymes, shaking experiments were performed in which very small glass beads having sieve numbers of 60/80

Table 18

Caseolytic activity of extract obtained by shaking cells with 5 mm glass beads at pH 6.6 and pH 7.0

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml extract pH 6.6 1 ml buffer pH 6.6 + 5 ml water	0.57	0.57	0.57	0.00
2	2 ml casein + 2 ml extr. pH 6.6 boiled 1 ml buffer pH 6.6 + 5 ml water	0.54	0.54	0.54	0.00
3	2 ml casein + 7 ml water 1 ml buffer pH 6.6	0.51	0.51	0.51	0.00
4	2 ml casein + 2 ml extract pH 7.0 1 ml buffer pH 7.0 + 5 ml water	0.71	0.72	0.71	0.00
5	2 ml casein + 2 ml extr. pH 7.0 boiled 1 ml buffer pH 7.0 + 5 ml water	0.69	0.67	0.65	0.00
6	2 ml casein + 7 ml water 1 ml buffer pH 7.0	0.69	0.69	0.69	0.00

Table 19

Caseolytic activity of extract obtained by shaking cells with 5 mm glass beads at pH 5.8 for 2 and 4 hours.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein 2 ml 2 hr. extract 1 ml buffer pH 5.8 5 ml water	0.49	0.52	0.61	0.12
2	2 ml casein 2 ml 2 hr. extr. boiled 1 ml buffer pH 5.8 5 ml water	0.47	0.47	0.47	0.00
3	2 ml 2 hr. extract 7 ml water 1 ml buffer pH 5.8	0.28	0.28	0.26	0.00
4	2 ml casein 2 ml 4 hr. extract 1 ml buffer pH 5.8 5 ml water	0.49	0.55	0.67	0.18
5	2 ml casein 2 ml 4 hr. extr.boiled 1 ml buffer pH 5.8 5 ml water	0.46	0.46	0.47	0.01
6	2 ml 4 hr. extract 7 ml water 1 ml buffer pH 5.8	0.32	0.31	0.31	0.00
7	2 ml casein 7 ml water 1 ml buffer pH 5.8	0.43	0.43	0.41	0.00

Table 20

Proteolytic activity of extract obtained by shaking cells with 5 mm glass beads at pH 5.8 for 4 hours using several types of peptones as substrates.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml trypticase + 1 ml extract 1 ml buffer pH 5.8 + 6 ml water	2.55	2.56	3.28	0.73
2	2 ml trypticase + 1 ml extr. boiled 1 ml buffer pH 5.8 + 6 ml water	2.52	2.50	2.55	0.03
3	2 ml tryptone + 1 ml extract 1 ml buffer pH 5.8 + 6 ml water	2.86	2.96	3.37	0.51
4	2 ml tryptone + 1 ml extr. boiled 1 ml buffer pH 5.8 + 6 ml water	2.87	2.85	2.89	0.02
5	2 ml proteose-peptone + 1 ml extract 1 ml buffer pH 5.8 + 6 ml water	2.30	2.35	2.80	0.50
6	2 ml proteose-peptone + 6 ml water 1 ml buffer pH 5.8 + 1 ml extr. boiled	2.39	2.37	2.32	0.00
7	2 ml tryptose + 1 ml extract 1 ml buffer pH 5.8 + 6 ml water	2.45	2.49	2.55	0.10
8	2 ml tryptose + 1 ml extr. boiled 1 ml buffer pH 5.8 + 6 ml water	2.43	2.44	2.44	0.01

were substituted for the large glass beads. When cell suspensions were shaken with these beads in the International Shaking Machine, active extracts could not be obtained. Microscopic examination of these shaken suspensions indicated that the small glass beads were also ineffective for disrupting the bacterial cells. Table 21 shows the results of titrations performed using a 2 and 4 hour extract obtained by shaking bacterial cells in the presence of the fine glass beads. The extract was tested for its caseolytic activity using casein as substrate buffered at pH 8.9. No increase in acidity of the test mixture was obtained.

Table 21

Caseolytic activity of extract prepared by shaking cells with small glass beads for 2 and 4 hours.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml 2 hr.extract 1 ml buffer pH 8.9 + 5 ml water	1.28	1.23	1.25	0.00
2	2 ml casein + 2 ml 2 hr.extr.boiled 1 ml buffer pH 8.9 + 5 ml water	1.25	1.22	1.26	0.00
3	2 ml casein + 2 ml 4 hr. extract 1 ml buffer pH 8.9 + 5 ml water	1.29	1.28	1.28	0.00
4	2 ml casein + 2 ml 4 hr.extr.boiled 1 ml buffer pH 8.9 + 5 ml water	1.27	1.27	1.27	0.00

9. Shaking cells in the high-intensity ultrasonic generator.

The disruption of bacterial cells by exposing them to high frequency oscillations has been successfully used as a means of obtaining various cell constituents. Hansen (1941), used extracts prepared by oscillating cells of Streptococcus cremoris and Streptococcus lactis according to the method of Chambers and Flosdorf (1936). He studied the influence of these extracts on the development of Lactobacillus casei. There was apparently no difficulty encountered in disrupting the cells by means of the high-frequency oscillations.

When cell suspensions of Lactobacillus casei were exposed to ultra-high-frequency vibrations, it was found that they resisted disruption by vibrations of 400 kilocycles for as long as fifteen minutes. Thus, it was found that crude enzyme containing extracts could not be produced from these organisms by using this method.

Microscopic examination of cell suspensions prepared after exposure to the high-frequency vibrations for 1, 2, 4, 8, and 15 minute intervals, showed no evidence of disruption of the cells. Studies of the effects of exposure to the vibrations for longer periods of time could not be carried out because the apparatus used created an excessive rise in temperature of the suspending medium. Determinations of the proteolytic activity of extracts produced by this method, uniformily showed the absence of proteases. The results obtained after five different intervals of shaking are shown in table 22. The titration figures indicate that for this series no activity against casein was present in any of the extracts used.

Table 22

Caseolytic activity of extracts prepared by exposing cell suspensions to ultra-high-frequency vibrations.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml 15 min. extract 1 ml buffer pH 5.8 + 5 ml water	0.46	0.47	0.47	0.01
2	2 ml casein + 2 ml 15 min. extr.boiled 1 ml buffer pH 5.8 + 5 ml water	0.43	0.41	0.41	0.00
3	2 ml casein + 2 ml 8 min. extract 1 ml buffer pH 5.8 + 5 ml water	0.44	0.46	0.45	0.01
4	2 ml casein + 2 ml 8 min.extr.boiled 1 ml buffer pH 5.8 + 5 ml water	0.43	0.44	0.43	0.00
5	2 ml casein + 2 ml 4 min. extract 1 ml buffer pH 5.8 + 5 ml water	0.44	0.44	0.44	0.00
6	2 ml casein + 2 ml 4 min.extr.boiled 1 ml buffer pH 5.8 + 5 ml water	0.43	0.43	0.44	0.01
7	2 ml casein + 2 ml 2 min. extract 1 ml buffer pH 5.8 + 5 ml water	0.45	0.46	0.45	0.00
8	2 ml casein + 2 ml 2 min.extr.boiled 1 ml buffer pH 5.8 + 5 ml water	0.43	0.42	0.42	0.00
9	2 ml casein + 2 ml 1 min. extract 1 ml buffer pH 5.8 + 5 ml water	0.44	0.43	0.43	0.00
10	2 ml casein + 2 ml 1 min.extr.boiled 1 ml buffer pH 5.8 + 5 ml water	0.42	0.43	0.40	0.00

10. Preparation of extracts by autolysis

The process of autolysis as a means of liberating bacterial cell constituents was successfully used by Berger, Johnson, and Peterson (1938a), in their study of the proteolytic enzymes of Leuconostoc mesenteroides. This method, although quite convenient to use and relatively simple to carry out, was not successful as a means of disrupting the cells of Lactobacillus casei. Cell-free extracts obtained by treating suspensions with 0.1N sodium hydroxide and incubating suitably, did not show any activity against casein, gelatin, or trypticase. The titration figures, for the various test mixtures used are recorded in table 23. No increase in acidity is noted, hence the extracts did not contain any proteinase or polypeptidase. On the other hand, when cells were allowed to incubate at pH 4.5 in the presence of Tween 80, the cell-free extract obtained contained a proteinase which hydrolysed casein. However, these extracts did not degrade either trypticase or gelatin. The titration figures are recorded in table 24. The extracts obtained by incubating cells at an alkaline pH in the presence of Tween 80 contained not only a proteinase active against casein but also a polypeptidase which caused the hydrolysis of trypticase. There was no apparent activity when gelatin was used as substrate. These results are given in table 25.

Table 23

Proteolytic activity of extracts obtained by autolysing cells at pH 7.3 for two weeks.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml extract 1 ml buffer pH 5.8 + 5 ml water	0.57	0.56	0.58	0.01
2	2 ml casein + 2 ml extr. boiled 1 ml buffer pH 5.8 + 5 ml water	0.56	0.54	0.54	0.00
3	2 ml gelatin + 2 ml extract 1 ml buffer pH 5.8 + 5 ml water	0.38	0.38	0.35	0.00
4	2 ml gelatin + 2 ml extr. boiled 1 ml buffer pH 5.8 + 5 ml water	0.35	0.35	0.35	0.00
5	2 ml trypticase + 2 ml extract 1 ml buffer pH 5.8 + 5 ml water	0.43	0.45	0.45	0.02
6	2 ml trypticase + 2 ml extr. boiled 1 ml buffer pH 5.8 + 5 ml water	0.41	0.40	0.38	0.00

Table 24

Proteolytic activity of extracts obtained by autolysing cells with Tween 80 at pH 4.5 for two weeks.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml extract 1 ml buffer pH 5.8 + 5 ml water	2.08	2.12	2.22	0.14
2	2 ml casein + 2 ml extr.boiled 1 ml buffer pH 5.8 + 5 ml water	2.09	2.10	2.11	0.02
3	2 ml gelatin + 2 ml extract 1 ml buffer pH 5.8 + 5 ml water	2.05	2.06	2.06	0.01
4	2 ml gelatin + 2 ml extr.boiled 1 ml buffer pH 5.8 + 5 ml water	2.01	2.01	2.02	0.01
5	2 ml trypticase + 2 ml extract 1 ml buffer pH 5.8 + 5 ml water	2.08	2.08	2.08	0.00
6	2 ml trypticase + 2 ml extr. boiled 1 ml buffer pH 5.8 + 5 ml water	2.06	2.06	2.06	0.00

Table 25

Proteolytic activity of extracts obtained by autolysing cells with Tween 80 at pH 8.9 for two weeks.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml extract 1 ml buffer pH 8.9 + 5 ml water	1.89	1.91	2.05	0.16
2	2 ml casein + 2 ml extr. boiled 1 ml buffer pH 8.9 + 5 ml water	1.87	1.86	1.84	0.00
3	2 ml gelatin + 2 ml extract 1 ml buffer pH 8.9 + 5 ml water	1.85	1.83	1.83	0.00
4	2 ml gelatin + 2 ml extr. boiled 1 ml buffer pH 8.9 + 5 ml water	1.83	1.83	1.83	0.00
5	2 ml trypticase + 2 ml extract 1 ml buffer pH 8.9 + 5 ml water	1.87	1.88	1.92	0.05
6	2 ml trypticase + 2 ml extr. boiled 1 ml buffer pH 8.9 + 5 ml water	1.84	1.83	1.83	0.00

11. Preparation of extract using 1M glycine

It was noticed by Cowles (1947), that when glycine solutions were added to suspensions of bacterial cells, there occurred an apparent lysis of the cells. Although for most of the work reported Escherichia coli and Bacillus mesentericus were the two organisms chiefly used, the author stated that many other bacteria were susceptible to the lytic action. He indicated that the method might be applied when experiments required the use of cell constituents, even including enzymes.

Experiments performed with this method, using a final concentration of 1M glycine and lactobacilli suspension, were in the main not successful. However, it was found that an extract active against casein was obtained when the cells were allowed to remain in contact with the glycine in the presence of an alkaline buffer. The extract obtained when the lysis was carried out at 30 C in the presence of toluene was only very slightly more active than that obtained by lysis at 6 C without toluene. The titration figures for these determinations are recorded in table 26. When these extracts were tested against dl-leucylglycine as substrate no activity could be discerned.

Microscopic examinations of gram stained smears of the treated cell suspensions showed that some of the cells had been lysed. However, most of the cells retained their typical morphological characteristics and the only change in them appeared to be that of altered reaction to the stain.

Table 26

Caseolytic activity of extract prepared by treating cells with glycine at 30 C and 6 C for 24 hours.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 2 ml 30 C extract 1 ml buffer pH 8.9 + 5 ml water	3.42	3.43	3.48	0.06
2	2 ml casein + 2 ml 30 C extr. boiled 1 ml buffer pH 8.9 + 5 ml water	3.42	3.43	3.42	0.00
3	2 ml casein + 2 ml 6 C extract 1 ml buffer pH 8.9 + 5 ml water	3.45	3.47	3.50	0.05
4	2 ml casein + 2 ml 6 C extr. boiled 1 ml buffer pH 8.9 + 5 ml water	3.40	3.40	3.40	0.00
5	2 ml casein + 1 ml 2 M glycine 1 ml buffer pH 8.9 + 6 ml water	3.35	3.34	3.34	0.00

12. Preparation of extract by using rennin

The action of rennin is ordinarily considered to be one in which the enzyme acts on casein, converting it to the relatively insoluble paracasein. This activity is optimum at pH 5.2 and decreases as the pH of the solution increases (Sumner and Somers 1947). However, under certain conditions, rennin can act as a proteolytic enzyme capable of degrading the casein to less complex nitrogen containing compounds.

Since rennin is frequently associated with Lactobacillus casei in the casein curd of certain cheeses, the somewhat improbable possibility that this enzyme could digest the bacterial cells with the concomitant liberation of the bacterial proteolytic endoenzymes was investigated.

When a suspension of Lactobacillus casei cells was allowed to remain in contact with a solution of rennin for 48 hours at pH 5.0, an extract was obtained which was capable of acting on casein at pH 5.8. A solution of rennin of the same concentration, treated the same way, showed some proteolytic activity but to a lesser extent. No activity against casein could be found when an extract obtained from the cells alone was used. The results of these determinations are recorded in table 27.

Microscopic examination of the rennin treated cells indicated that extensive lysis did not occur.

Table 27

Caseolytic activity of extract prepared by treating cells with rennin at pH 5.0.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 1 ml rennin extract 1 ml buffer pH 5.8 + 6 ml water	0.65	0.67	0.75	0.10
2	2 ml casein + 1 ml rennin extr.boiled 1 ml buffer pH 5.8 + 6 ml water	0.45	0.45	0.46	0.01
3	2 ml casein + 1 ml rennin 1 ml buffer pH 5.8 + 6 ml water	0.40	0.42	0.44	0.04
4	2 ml casein + 1 ml rennin boiled 1 ml buffer pH 5.8 + 6 ml water	0.39	0.39	0.39	0.00
5	2 ml casein + 1 ml cell extract 1 ml buffer pH 5.8 + 6 ml water	0.44	0.44	0.44	0.00
6	2 ml casein + 1 ml cell extr.boiled 1 ml buffer pH 5.8 + 6 ml water	0.42	0.43	0.42	0.00
7	1 ml rennin-cell mixture + 8 ml water 1 ml buffer pH 5.8	0.67	0.65	0.67	0.00
8	1 ml rennin + 1 ml buffer pH 5.8 8 ml water	0.25	0.25	0.24	0.00
9	1 ml cell suspension + 8 ml water 1 ml buffer pH 5.8	0.31	0.31	0.30	0.00

13. Preparation of extract by grinding with solid carbon dioxide

Freezing bacterial cells at extremely low temperatures in order to disrupt them has not been used very extensively in the past. Tarnanen (1930), attempted to obtain a proteolytic extract by alternate freezing in liquid air followed by rapid thawing of the frozen bacterial cell mass at 50 C. Extracts prepared in this way showed either a very weak proteolytic activity or were not active at all. It is known that long, sharply pointed ice crystals are formed within the cells when they are slowly frozen. However, when the cells are rapidly frozen, these crystals do not form, and consequently the cells are not ruptured. Bacterial cells which have been rapidly frozen at very low temperatures should be somewhat easily disrupted by grinding since it is probable that the individual cells are rendered extremely brittle.

When suspensions of Lactobacillus casei were frozen in dry ice and then ground with it, it was possible to obtain cell-free extracts which were weakly proteolytic. Unfortunately, not all of the extracts produced by this method showed activity, and the results obtained with a given extract were not reproducible.

D. Action of extracts prepared in the Mickle Tissue Disintegrator.

1. Disruption of cells

It was found that successful preparations of crude cell-free extracts could be procured from Lactobacillus casei 318, when suspensions of these cells were shaken in the Mickle Tissue Disintegrator. The progress of cell

disruption was followed by examination of stained films of the cell suspensions before and after shaking. A typical example of the results obtained is shown in the following photomicrographs and electronographs. Figure 2 is a photomicrograph of a cell suspension of Lactobacillus casei 318 prepared for treatment in the Mickle Tissue Disintegrator. The cells shown, have the typical morphology of this species. No extraneous amorphous material can be observed. Figure 3, shows a cell suspension photographed after 20 minutes of shaking. Here, only a few cells can be seen which are intact. Most of the cells have been destroyed, and the preparation essentially consists of a large amount of cell debris. A more detailed view of the disintegration process was obtained by observation of the cell suspensions with the electron microscope. Figure 4 is an electronograph of an unshaken cell suspension. The typical morphology and internal structure of the cells can be seen. In figure 5 is shown the results obtained using a shaken cell suspension. This electronograph shows some empty cell walls with a large amount of extruded protoplasm. When preparations of this type were suitably treated to remove cell debris, the cell-free extracts obtained were usually found to be proteolytically active.

2. Determination of optimum time for shaking

The cell free extract which was obtained by centrifugating the shaken cell suspensions was active at pH 5.8 and pH 8.9. These results agreed with those obtained when extracts obtained by other methods were used. When cell suspensions were shaken for 5 minutes, it was found by microscopic study, that approximately one-third of the cells were destroyed, while after shaking

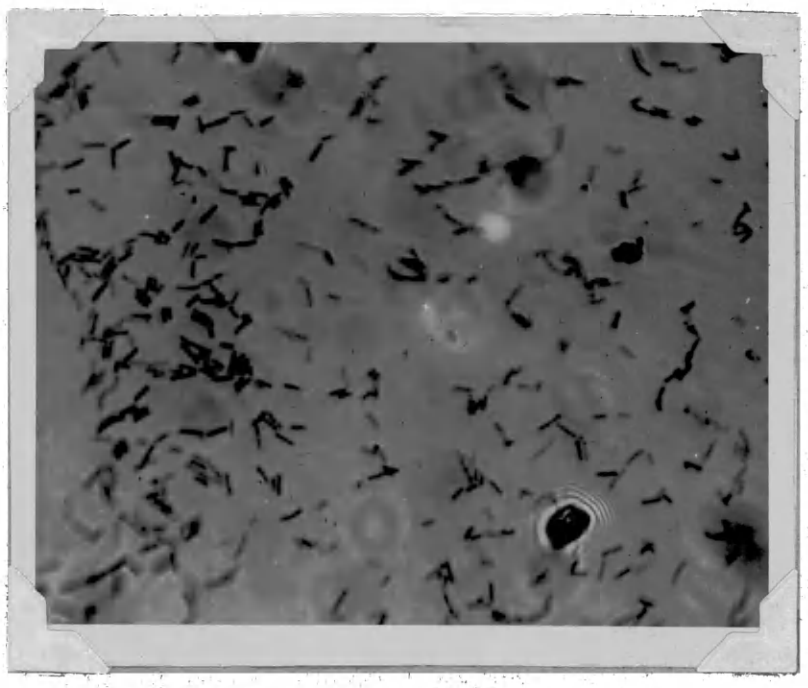

Figure 2. Cells of Lactobacillus casei 318 before shaking in the Mickle Tissue Disintegrator. X 970

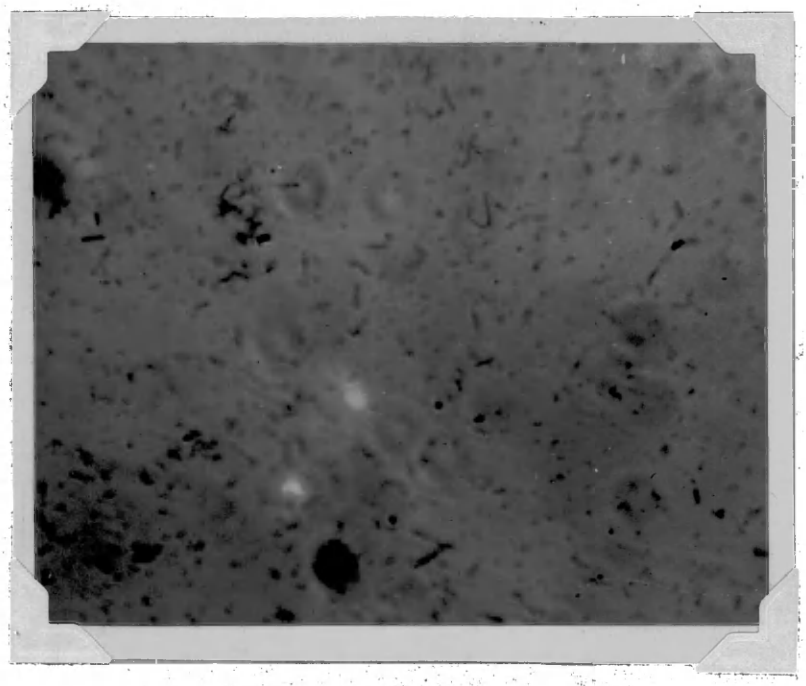

Figure 3. Cells after shaking for 20 minutes in the Mickle Tissue Disintegrator. X 970

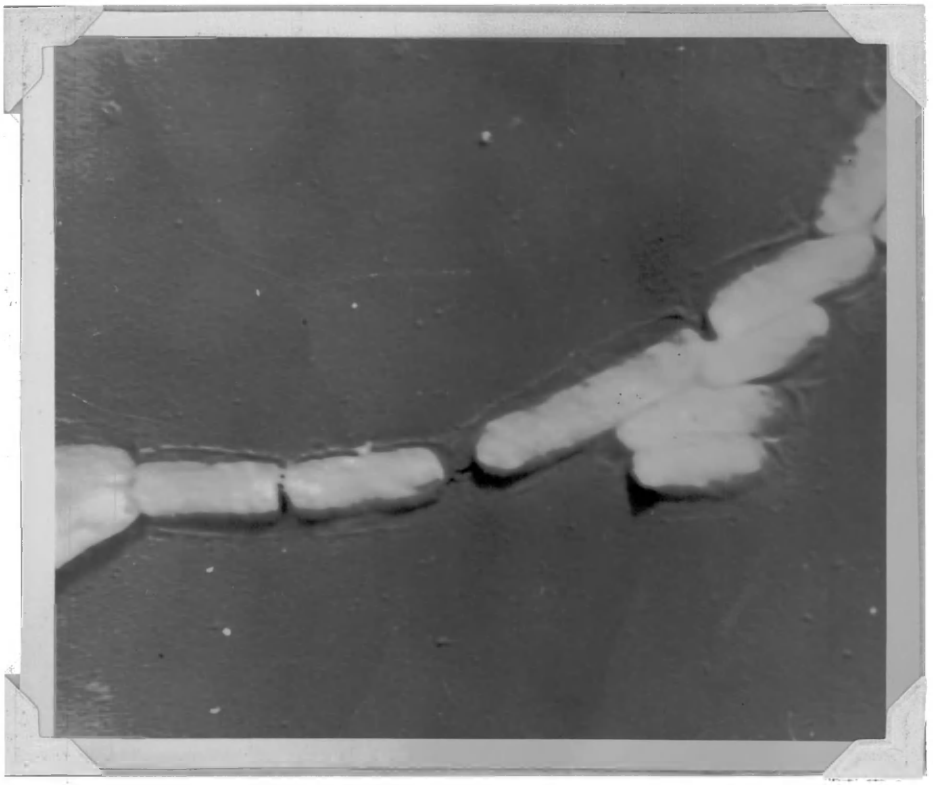

Figure 4. Electronograph of suspension of Lactobacillus casei 318 before shaking X 10,000

Figure 5. Electronograph of shaken suspension of Lactobacillus casei 318 X 10,000

for 25 minutes almost all of the cells were disintegrated. The results of determinations of proteolytic activity of extracts obtained after 5 and 25 minutes of shaking is shown in table 28. The titration figures indicate that at an alkaline pH some of the enzyme was destroyed by the longer shaking time, but the reverse was true when the extract was prepared at an acid pH. It appeared that more enzyme was liberated, since a greater final acidity was found in the test mixture.

3. Determination of extract activity against gelatin

When the extract was tested using gelatin as substrate, it was found to be relatively weakly active. However, this substrate was acted on, as was found in the case of casein, at both acid and alkaline pH values. The extent of hydrolysis, as measured by the increase in acidity of the test mixture, was always less when gelatin was used as substrate, than when casein was used. Table 29 shows the titration values obtained when the extract was tested against gelatin at several pH levels.

4. Influence of glutathione on the hydrolysis of casein

Glutathione may act either as an activator or inhibitor of certain enzymes. Thus, Willstätter and Grassmann (1924), found that glutathione would activate the proteolytic enzyme, papain. Bromelin was found by Bergmann, Fruton, and Fraenkel-Conrat (1937), to be activated by this compound also.

When the extract obtained from Lactobacillus casei 318 was treated with solutions of glutathione for 2 to 3 hours and then allowed to react with casein, no increase of activity was found. In some instances however,

Table 28

Caseolytic activity of extracts prepared in Mickle Tissue Disintegrator at pH 5.8 and pH 8.9.

Tube No.	Test Mixture	ml KOH used Hours 0	1	24	Increase in acidity
1	2 ml casein + 1 ml 5 min. extr. 1 ml buffer pH 5.8 + 6 ml water	0.52	0.53	0.63	0.11
2	2 ml casein + 1 ml 5 min. extr.boiled 1 ml buffer pH 5.8 + 6 ml water	0.51	0.52	0.52	0.01
3	2 ml casein + 1 ml 25 min.extract 1 ml buffer pH 5.8 + 6 ml water	0.56	0.59	0.70	0.14
4	2 ml casein + 1 ml 25 min.ext.boiled 1 ml buffer pH 5.8 + 6 ml water	0.51	0.53	0.51	0.00
5	2 ml casein + 1 ml 5 min.extract 1 ml buffer pH 8.9 + 6 ml water	1.59	1.64	1.72	0.13
6	2 ml casein + 1 ml 5 min.extr.boiled 1 ml buffer pH 8.9 + 6 ml water	1.54	1.56	1.54	0.00
7	2 ml casein + 1 ml 25 min. extract 1 ml buffer pH 8.9 + 6 ml water	1.65	1.67	1.75	0.10
8	2 ml casein + 1 ml 25 min.extr.boiled 1 ml buffer pH 8.9 + 6 ml water	1.64	1.61	1.63	0.00

Table 29

Gelatinolytic activity of extracts at various pH values.

Tube No.	Final pH	Increase in acidity ml KOH after 24 hours
1	9.1	0.09
2	8.2	0.06
3	6.9	0.00
4	5.9	0.00
5	5.0	0.05
6	4.0	0.00

it was found that the activity of the extract was depressed when in the presence of the glutathione. Table 30 shows the results of the determinations of caseolytic activity of glutathione treated extracts compared with the activity of untreated extract. The titration figures indicate that for the proteinase present in the extract, glutathione acts as a partial inhibitor of activity.

5. Influence of various ions on the proteolytic activity of cell-free extracts

Various metallic ions have been shown to be activators for certain types of proteinases. Thus, Weil and Kocholaty (1937), reported the activation of a proteinase obtained from an anaerobe. These workers stated maximum activity of the proteinase was exhibited under anaerobic conditions

Table 30

Caseolytic activity of untreated and glutathione treated extract.

Tube No.	Test Mixture	Increase in acidity after 24 hours	
		pH	ml KOH
1	untreated extract + casein	6.0	0.28
2	glutathione treated extract casein	6.1	0.24
3	untreated extract + casein	8.9	0.32
4	glutathione treated extract casein	8.7	0.26
5	glutathione + caseine alone	6.1	0.00
6	glutathione + casein alone	8.7	0.00
7	glutathione treated extract alone	6.0	0.01
8	glutathione treated extract alone	8.5	0.03

in the presence of very low concentrations of ferrous, manganous, nickle, cupric, and even cobalt ions. Information relative to the effect of various inorganic ions on the activity of proteinases obtained from lactobacilli could not be found in the literature.

In order to test the effect of various inorganic ions on the proteolytic activity of the proteinases found in crude extracts of Lactobacillus casei 318, the extract was treated with suitable quantities of each of the following freshly prepared salt solutions. The salt solutions used were: ferrous sulfate, manganous sulfate, magnesium sulfate, cobaltous chloride and sodium cyanide.

When the cell-free extract was treated with ferrous ions and then allowed to react with casein, it was found that dilute concentrations of these ions apparently increased the activity of the extract. This increase in activity was noted however, only when the extract was tested at the alkaline pH range. A decrease in activity was observed when the ferrous ion treated extract was tested at an acid pH value. Manganous ions on the other hand seemed to increase the extract of activity at the acid pH value, while decreasing the activity at some alkaline values. The relative increase or decrease of the caseolytic activity of the cell-free extract after treatment with the various ions is shown in table 31.

Sodium cyanide acted as an inhibitor when the extract - substrate mixture was tested at pH 8.9 and 5.8. At pH 8.0 the inhibition of the caseolytic activity by this substance was not complete, having decreased the titration figure by 80 per cent. Near neutrality only a relatively small inhibition was noted.

Table 31

Influence of various inorganic ions on the relative activation on inhibition of caseolytic activity of extract

pH of mixture	ml KOH used per 10 ml test mixture	Relative per cent increase or decrease of extract activity - casein substrate				
		Activator added				
	Extract not treated	$FeSO_4$ 0.002M	$MnSO_4$ 0.002M	$MgSO_4$ 0.008M	$CoCl_2$ 0.0008M	NaCN 0.004M
8.9	0.40	+ 25	- 75	+ 25	0	- 100
8.0	1.00	+ 90	0	0	-40	- 80
6.9	0.25	+ 20	- 20	- 15	-60	- 20
5.8	0.50	- 80	+ 80	- 24	-100	- 100
5.0	0.00	0	0	0	0	0

The cell-free extract when tested without any previous treatment generally exhibited feeble activity against gelatin as substrate. It was noted in the experiments conducted with the various inorganic activators used, that they generally did not effect much change in the activity of the extract in its action against gelatin. Nevertheless, it was found that dilute solutions of cobalt ions apparently increase the activity of the extract. The same observation was made for solutions of sodium cyanide All of the other ions either inhibited the activity of the extracts or did not have any effect. The relative activity of the untreated ions, at various pH values is shown in table 32.

Table 32

Relative activation or inhibition of gelatinolytic activity of cell-free extract by various inorganic ions

pH of mixture	ml KOH used per 10 ml test mixture	Relative per cent increase or decrease of extract activity - gelatin substrate				
		Activator added				
	Extract not treated	$FeSO_4$ 0.002M	$MnSO_4$ 0.002M	$MgSO_4$ 0.008M	$CoCl_2$ 0.0008M	NaCN 0.004M
9.0	0.32	- 68	- 100	- 100	≠ 56	- 100
8.0	0.21	- 100	- 5	- 100	≠ 43	≠ 43
7.0	0.00	0	0	0	0	0
5.9	0.00	0	0	0	0	0
5.0	0.18	- 100	- 100	- 100	- 100	- 100
4.0	0.00	0	0	0	0	0

6. Determination of extract activity against peptones

When cell-free extracts were tested against various peptones, it was found that a relatively good activity against these substrates was elicited. Most of the extracts tested consistently caused the hydrolysis of trypticase and tryptone, while the action against Bacto-peptone was found to be extremely variable. Tryptose and proteose-peptone were not attacked. It was found that the pH of the reacting solution greatly influenced the extent of hydrolysis of the particular peptone substrate used. The titration figures illustrating the results which were obtained in these experiments are recorded in table 33. It can be seen that there apparently were for the polypeptidases present in the crude extract, two pH values at which maximum activity could be obtained. For the substrate trypticase the greatest activity was obtained at pH 5.8, with a second value obtained at the alkaline value of pH 8.0.

Table 33

Proteolytic activity of extract using several peptones as substrates at different pH values.

pH of mixture	ml of KOH used per 10 ml of reaction mixture		
	Trypticase	Tryptone	Peptone
9.5	0.02	0.21	0.00
8.9	0.05	0.80	0.00
8.0	0.93	0.50	0.00
7.2	0.30	0.00	0.00
6.6	0.41	0.33	0.11
5.8	1.00	0.70	0.22
5.0	0.52	0.56	0.06
4.5	0.21	0.26	0.00
4.0	0.03	0.05	0.00

Tryptose on the other hand, was acted on best at pH 5.8 and pH 8.9. However, the extent of hydrolysis of this substrate appeared to be less at these optimum pH values than was observed for trypticase. While the extract showed activity when it was tested against peptone, it was much less extensive, and was found to occur best at pH 5.8. In the experiments performed by Tarnanen (1930), it was found that the cell-free extract split Witte's peptone at an optimum value of pH 5.8. The activity of the extract fell rather rapidly above pH 6.6. This author did not test his extract above pH 7.7.

7. Determination of extract activity against various chemically defined peptides

Tarnanen (1930), working with glycerine extracts of Bacterium casei epsilon, found that not only did it attack Witte's peptone, but could also cause the hydrolysis of leucylglycine as well as glycylalanine. The activity of the extract against other peptides was not investigated. Since the extracts obtained in the work reported here could hydrolyse various peptones, which are ill defined mixtures of peptides with other nitrogen containing compounds, it was thought that the content of peptidases in the extract could be more clearly elucidated if the extract was tested against several chemically defined peptides.

For the detection of aminopolypeptidase, dl-leucylglycylglycine was used as substrate. It was found that the extract acted on this substance only at pH 8.0. At this pH value the average extent of hydrolysis, as calculated from the increase of acidity of the test mixture after 24 hours,

was found to be 29.1 per cent. No hydrolysis of this tripeptide could be detected at other more acid pH levels.

In order to detect the presence in the extract of carboxypeptidase the extract was tested against chloracetyltyrosine. This substrate was used since carboxypeptidase will act on polypeptides containing a free carboxyl group, but will not act on such compounds if a free amino group is present. When the cell-free extract obtained from Lactobacillus casei 318 was allowed to act on chloracetyltyrosine, no activity was detected. The presence of this enzyme in other bacteria apparently has not been detected to date, since no reference for this activity has been found in the literature.

In addition to hydrolysing the tripeptide the extract obtained in these experiments acted on chemically defined dipeptides.

When the extract was allowed to act on dl-alanylglycine and d-leucyl-l-tyrosine, it was found that maximum activity occurred between pH 5.0 and pH 5.6. However, at these optimum pH values, only 20 per cent of the dl-alanylglycine was found to be hydrolysed. There was a gradual decrease in the extent of hydrolysis of this substrate when it was tested at pH values on either side of the optimum. On the other hand, d-leucyl-l-tyrosine was hydrolysed only at the pH range 5.0 to 5.6. The greatest amount of hydrolysis of this substrate was also found to be 20 per cent of the original concentration. The results of these determinations are recorded in table 34.

The results of the determinations using l-leucylglycine and glycyl-l-leucine are shown in table 35. When the extract was tested against glycyl-l-

Table 34

Determination of extract activity using dl-alanylglycine and d-leucyl-l-tyrosine as substrates.

Per cent hydrolysis of dipeptides			
pH	dl-alanylglycine	pH	d-leucyl-l-tyrosine
9.1	4	8.0	0
8.3	6	7.2	0
7.2	4	6.8	0
6.8	6	6.1	0
6.1	16	5.6	20
5.6	20	5.0	16
5.0	20	4.5	0
4.5	4		

Table 35

Determination of extract activity using glycyl-l-leucine and l-leucylglycine

Per cent hydrolysis of dipeptides			
pH	glycyl-l-leucine	pH	l-leucylglycine
8.9	0	8.9	13
8.0	5	8.0	13
7.5	7	7.8	31
6.9	10	7.6	31
6.1	23	7.2	50
		6.2	21
5.6	33	5.6	12
5.0	100	4.8	9
4.5	5		

leucine, the optimum range for hydrolysis was again found to be pH 5.0 to 5.6. In this case, it was observed that all of the substrate was hydrolysed at the lower value, while at the upper limit one third of the original amount of substrate was hydrolysed. When the extract was tested using l-leucylglycine the greatest activity was found to occur at pH 7.2 to 7.8. At pH 5.6 only 12 per cent of the original concentration of substrate was found to be hydrolysed.

IV

DISCUSSION AND SUMMARY

The true lactic acid bacteria are markedly active as fermenters of carbohydrates. Ample evidence exists that they may be actively proteolytic when suitable experimental conditions are chosen for the liberation and action of such enzymes. In addition to the empirical observations made by von Freudenreich (1895), other more exact data support this view. Thus, Orla-Jensen (1919), recorded the fact that many of the rod-shaped lactic acid bacteria were capable of increasing the soluble nitrogen content of cultures as much as 36 per cent. Also, Peterson, Pruess, and Fred (1928), found substantial increases in the soluble nitrogen content of cultures of various lactic acid forming bacteria.

Thus, it was expected that the strains of Lactobacillus casei which were studied in this work, under proper cultural conditions would hydrolyse casein. That this was indeed the case was shown by the findings obtained by the analyses of the calcium carbonate-skim milk cultures. Everyone of the strains was found to be capable of degrading the casein present in the original medium. This decomposition was manifested by a decided increase of the soluble nitrogen content in the culture filtrate. Of the organisms tested, the most active was found to be strain 318, since it caused the soluble nitrogen content to increase 1.93 mg per ml of culture filtrate. The least active organism was found to be strain 322, which increased the soluble nitrogen content only 0.04 mg per ml of filtrate.

On the basis of the above findings, strain 318 was selected as the cell source from which the crude endo-cellular enzyme extracts were prepared. This organism, although originally listed by Orla-Jensen as a strain of plantarum, was considered by Tittsler to be actually a strain of casei. Experience with the reactions of this organism during the work reported here, also indicated that it is casei rather than plantarum. It was found that this strain was unable to ferment melibiose and of course that it was actively proteolytic.

The fact that Lactobacillus casei cells were resistant to destruction by various physical and chemical agents, necessitated an investigation of methods which might be used to disrupt the cells. It is obvious that successful studies on the activity of endoenzymes cannot be carried out unless suitable procedures for the preparation of cell-free juices are used. In the studies presented here, the efficiency of a method as a means of preparing extracts of cells was followed by microscopic observation of the morphology of the treated bacterial cells. The proteolytic activity of a given extract was also used as a measure of the suitability of a given procedure for the preparation of a cell-free extracts.

Although grinding procedures have been successfully used as a means of obtaining cell-free juices (Wiggert, Silverman, Utter, and Werkman, 1939), (McIlwain, 1948), it was found that they were not satisfactory when applied to the preparation of extracts from cells of Lactobacillus casei 318. Thus, grinding cells in the frozen state with solid carbon dioxide, or

alumina, or sand was generally unsuccessful. Also unsuitable were: alternate freezing and thawing; shaking in the International Shaking Machine with small pavement marking glass beads; shaking at 400 kilocycles in a high-intensity ultrasonic generator; and autolysing cell suspension at 37 C and pH 7.3. Repeated experiments using the above listed methods as devices for obtaining proteolytically active extracts showed that they were not useful.

Although alumina ground cells were found not to yield active extracts, eluting the alumina-cell mass with M/15 phosphate solution produced a solution which showed some activity against casein, gelatin, and trypticase at pH 8.9. Apparently the alumina adsorbed the proteinases and polypeptidase during the grinding process.

The glycerol extract of Lactobacillus casei 318 was found to be capable of hydrolysing casein, trypticase, tryptone, proteose-peptone and tryptose. This is essentially in agreement with the results obtained by Tarnanen (1930), when he tested glycerol extracts of Bacterium casei epsilon (Lactobacillus helveticus, in the present notation as used in Bergey's Manual, sixth edition).

The successful preparation of proteolytically active extracts by shaking with glass beads in the International Shaking Machine, was found to depend on two factors, namely, the reaction of the medium in which the cells were suspended, and the size of the glass beads used. Thus it was found that very small pavement marking beads were ineffective agents for cell disruption, while glass beads approximately 5 mm in diameter were found to be effective. The extracts which were obtained after 2 and 4

hour periods of shaking at pH 8.9 or pH 5.8 were capable of hydrolysing casein at both of these pH values, but no activity of extracts prepared at pH 6.6 or pH 7.0 could be detected. It was also found that the extract prepared only at pH 5.8 could hydrolyse the various peptone substrates tested. Therefore, it is thought that the proteinase is not destroyed at either pH 5.8 or pH 8.9, but that the polypeptidases which are liberated are sensitive to the higher value.

In view of the fact that usual autolysis procedures were ineffective for the production of extracts of Lactobacillus casei 318, the inclusion of some agent which might facilitate lysis of the cells was felt to be justified. Tween 80, a surface active agent, was found to aid in the production of active extracts. Thus, it was found that at an acid pH, a caseolytic proteinase only was obtained, while at an alkaline pH both a proteinase and polypeptidase could be detected. Paradoxically, although proteolytically active extracts were obtained by means of Tween 80, this agent did not seem to cause any great amount of disintegration of the cells. There exists the possibility that the surface active agent allowed the liberation of the endocellular enzymes, not by any obvious attack on the morphology of the cells, but by bringing about subtle changes in the permeability of the cell wall. Data to support this contention cannot be found in the literature and valid experimental evidence is at present lacking.

The use of glycine as an agent for the liberation of endocellular enzymes does not appear to have been applied heretofore. Cowles (1947), however, suggested that this compound might be successfully utilized when work with cell constituents became necessary. The application of this method

for obtaining proteolytically active extracts from the lactobacillus used in the work presented here, met with indifferent success, since preparations of uniform activity were not obtained. The nature of the action of glycine on bacterial cells is unknown, and microscopic examination of glycine treated cells did not indicate any extensive physical breakdown of the cells.

The extracts obtained from the bacterial cells which had been treated with rennin were found to increase the acidity of extract-substrate test mixtures two and one-half times that found when rennin was used alone. This fact indicates that a proteinase was liberated from rennin treated cells. Amunstad (1950), reported also that the proteolytic activity of glycerol extracts prepared from Streptobacterium casei using casein as a substrate was enhanced when rennin was present in the test mixture. While it appeared that rennin could act in some way on the cells of Lactobacillus casei 318 to liberate a proteinase, it was also found that the bacterial cells were not without effect on the rennin. This was illustrated by the fact that the milk clotting power of the rennin, which had been in contact with the bacterial cells, was diminished by approximately 50 per cent. This phenomenon may possibly be explained by assuming that some of the rennin was adsorbed on the surface of the bacterial cells during the period of contact. Upon centrifugation of the rennin-cell mixture to obtain a cell-free extract, this adsorbed rennin was removed, thus effectively lowering the concentration of rennin which remained in the solution. Thus, the milk clotting power of the rennin, as measured by the method of Kunitz (1935), was in reality based on a quantitative change rather than a qualitative one.

The Mickle Tissue Disintegrator was found to be superior to all the other methods used for the preparation of proteolytically active extracts from the cells of Lactobacillus casei 318. This instrument was simple to operate, and yielded a high proportion of active preparations.

When these extracts were tested against casein, it was found that activity was exhibited against this substrate at both acid and alkaline values. The caseolytic activity appeared to function at pH 5.8 to 6.0 and pH 8.0 to 8.9. It was also found that the extract was capable of hydrolysing gelatin. However, in the case of this substrate some activity was noted at pH 5.0 but greater activity was found at pH 8.2 and 9,1. The finding that the pH of activity of the extract changed when different protein substrates were used agrees with the observations made for proteinases obtained from other sources (Sumner and Somers, 1947).

Although glutathione is an important activator for several proteolytic enzymes obtained from higher plants, it was not found to act in this capacity when tested with the proteinase present in the crude extracts of Lactobacillus casei 318. There appeared to be a slight depressant action of this substance on the caseolytic activity.

For the most part, the metallic ions which were tested either had no effect on the action of the extract or depressed the activity. When casein was used as the substrate, ferrous ions seemed to increase the activity of the extract at pH range of 8.0 to 8.9; but at pH 5.8 there was inhibition. When gelatin was used as the substrate most of the ions used acted as inhibitors of activity except cobalt, which seemed to increase the enzyme activity when it was tested at pH 8.0 to 9.0. While

some bacterial proteinases may be secreted by the cell into the surrounding medium, Lactobacillus casei 318 seems to retain most of the proteinases within the cell wall. It is generally considered unlikely that protein molecules such as casein may pass freely in and out of the cell through the cell wall. Hence the following hypothesis is presented: Lactobacillus casei 318 actually secretes some proteinases which, however, attain significant concentrations only in the immediate neighborhood of the cell, where they may be so firmly adsorbed to the cell surface, that they cannot be removed unless the cell is disintegrated. It is also possible that the hydrolytic action of the enzymes is exerted on the molecules of substrate immediately surrounding the cell. The end products of smaller molecular size produced by this reaction are then able to pass into the cell where they are further utilized.

When the extract was tested against various peptones, it was found that there were two pH ranges at which hydrolysis occurred. Thus, when trypticase and tryptone were used as substrates, the extract exhibited activity at pH 5.0 to 5.8 and pH 8.0 to 8.9. When, on the other hand, Bacto peptone was used as substrate, some activity was noted between pH 5.0 to 6.6. The finding that the activity of the extract on peptones shows rather wide variance may possibly be ascribed to the fact that these substances varied in composition. Trypticase and tryptone are derived by hydrolysis of casein, while Bacto peptone is obtained by hydrolysis of gelatin, and it seems feasible to conclude that since the proteinase present in the extract is more active against casein the polypeptidase present may be more active against the peptones derived from it than from peptones derived from other proteins.

Although the extract was found to contain peptidases active against several types of peptides, no carboxypeptidase was found. This may be explained perhaps, by the fact that the substrate used for the detection of this enzyme was one which does not occur naturally, and consequently if carboxypeptidase was present it could not act on the substrate. Carboxypeptidase has not been found in bacteria so far; but the number of species which have been investigated is, admittedly, small. However, the extract exhibited activity against other types of chemically defined peptides. Thus, activity was exhibited against dl-alanylglycine between pH 5.0 and 6.1, and against d-leucyl-ltyrosine at pH 5.0 to 5.6. The best activity against glycyl-l-leucine was found to occur between pH 5.0 and 6.1. Berger, Johnson, and Peterson (1938b), reported that many of the peptidases obtained from various species of bacteria showed maximum activity between pH 8.0 to 9.0. In this respect the peptidases obtained from Lactobacillus casei 318 differed from those obtained by these authors. The same workers reported that they obtained peptidases from "Lactobacillus pentosus" which exhibited maximum activity at pH range of 5.5 to 6.0. The results obtained in the work presented here agree with the latter observation. "Lactobacillus pentosus" is a peculiar strain of Lactobacillus plantarum, which in turn is very closely related to Lactobacillus casei. Hence, it is not surprising that similar results were obtained.

The observations on the proteolytic activities of Lactobacillus casei 318 and the results obtained using cell-free extracts indicate that these organisms contain a rather wide spectrum of proteolytic enzymes. Under suitable conditions, these enzymes can be liberated from the bacterial

cells as a crude mixture. The activity of the crude cell-free extracts on various substrates indicates that there are present proteinases, polypeptidases, and dipeptidases. No carboxypeptidase activity of the extracts can be detected.

V

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