lowered the lysine content of the food. Fermentation of materials low in lysine would seem more advantageous. Completely new high lysine types of fermented foods might, of course, be made with lysine excreting mutants.

The lysine excreting strains were indistinguishable from the wild types in growth and rate of fermentation. The mutants, being spontaneous and easy to select and detect, can be obtained from current wild types to avoid possible taste differences from new strains.

To our knowledge high lysine producing mutants have not previously been used in natural food fermentations. We have obtained substantial increases in free lysine excretion with spontaneous mutants of lactobacilli. Mutants excreting even more lysine, or other amino acids, might be obtained either by the methods we describe, or other methods. Before any mutants are used, however, it is important to know if they are producing any unwanted metabolites. Furthermore, care must also be exercised to assure that there will not be a nutritional imbalance of some amino acids.

Our studies demonstrate the relatively simple procedures needed to allow selection of spontaneous mutants of high-lysine producing bacteria that could be used in natural fermentation of foods. Similar application of these techniques to find yeasts and molds used in fermentation that can excrete large amounts of essential amino acids should be possible.

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Hydrolysis of Milk Proteins by Bacteria Used in Cheese Making

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Literature pertaining to the role of lactic acid producing bacteria and *Brevibacterium linens* and *Propionibacterium shermanii* in the hydrolysis of proteins in cheese is reviewed. Particular emphasis is given to our work on the cellular location and proteolytic activity of selected strains of lactic streptococci. Cells of *Streptococcus lactis* C2 and *Streptococcus cremoris* ML1 harvested from Elliker's broth during late log, stationary, and early death phases of growth were ruptured in the Eaton press and fractionated into soluble, membrane, and ribosomal fractions by differential centrifugation. Enzyme activity on selected dipeptides and whole casein was determined on each cellular fraction. The soluble fraction contained the highest activity while the ribosomal fraction contained the lowest enzyme activity. *S. lactis* C2 had higher enzyme activity than *S. lactis* ML1. Activity on whole casein was reduced drastically in cells harvested during early death phase as compared to cells harvested during late log phase of growth while dipeptidase activity was relatively constant.

The manufacture of high quality cheese is primarily dependent on controlled microbial fermentation of milk constituents. Breakdown of lactose, fat, and protein is in one state or another necessary to obtain the desired curd type, texture, body, and flavor. Considerable attention has been directed to the importance of amino acids in cheese. Amino acids and peptides without doubt have an influence on cheese flavor (Storgårds and Lindquist, 1953; Harper, 1959). Proteins induce little, if any, flavor but are important for the body and texture.

The breakdown of proteins, primarily caseins, in cheese could be caused by rennet, milk proteinase, and microbial proteinases and peptidases. The milk proteinase seems to have only limited influence on cheese ripening (Zittle, 1965; Peterson, 1972). Only small amounts are found in milk and the presence of residual enzyme in pasteurized milk is questionable (Stadhouders, 1959; Kaminogawa et al., 1969; Chen and Ledford, 1971).

The effect of rennet on casein hydrolysis, besides the specific effect on κ-casein, has been a source of controversy. Stadhouders (1959) states that rennet is only of minor importance to casein degradation. On the other hand, Mabbitt et al. (1955) and Reiter et al. (1966) have characterized rennet as the most important source of proteolytic enzymes in cheese making. Others have claimed that rennet has minimum influence on cheese flavor because the products of casein hydrolysis by rennin are mainly protein fragments, and that only small amounts of amino acids are found (Sato and Ohmiya, 1966; Ohmiya and Sato, 1969a,b; Reiter and Sharpe, 1971). In a recent

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study, Dulley (1974) concludes that rennet could cause hydrolysis of casein to peptides and amino acids if present in high concentrations. The proteolytic effect in cheese was found to be minor compared to that of starter organisms due to low levels of rennet retained in the curd.

In the following, a brief discussion will be given on proteolysis of milk proteins by some common bacteria used in cheese making. Several review articles have been published on the general topic of cheese flavor and ripening (Mulder, 1952; Mabbitt, 1961; Marth, 1963; Forss and Patton, 1966; Reiter et al., 1966; Scott, 1967; Fryer, 1969; Reiter and Sharpe, 1971; Dwivedi, 1973; Stadhouders, 1974; Langsrud and Reinbold, 1973).

Already in 1914, Hart et al. suggested a possible role of lactic streptococci in cheese proteolysis. A more conclusive relationship has been reported in later years. Proteolysis of aseptic curd by single strain cultures of *Streptococcus lactis*, *S. cremoris*, and *S. diacetilactis* was found to occur with nonprotein nitrogen (NPN) and amino nitrogen (AN) as products (Reiter et al., 1966). Upon incubation of *S. lactis* in skim milk at 30 °C, Collins and Nelson (1949) observed an initial rapid increase followed by a more gradual increase in NPN content. Dolezalek (1966) incubated pure cultures of *S. lactis*, *S. cremoris*, *S. diacetilactis*, *Lactobacillus helveticus*, and *L. casei* in sterile skim milk at optimum temperature for 30 days. The lactobacilli generally had higher proteolytic activity and produced large amounts of amino acids. The amino acids produced in largest amount under the influence of either streptococci or lactobacilli were glutamic acid, proline, lysine, and leucine. Proteolysis by *S. diacetilactis* was similar to that of the lactobacilli. It was concluded that all organisms could exert an important role in proteolytic breakdown during cheese ripening.

In a comparative study of different species of streptococci and lactobacilli grown in milk, Miller and Kandler (1967) found that dialyzable nitrogen increased about 1–2% by the action of streptococci. In contrast, the increase caused by the thermophilic lactobacilli was 4–5%. Amino acid nitrogen (AN) made up the major part of the dialyzable nitrogen released by the lactobacilli. On the other hand, ammonia nitrogen was released in the greatest amount by the streptococci. A study comparing the release of amino acids from milk cultures by *L. casei* and *L. lactis* is reported by Pande et al. (1970). Rapp (1969) observed that hydrolysis of casein was highest by *L. acidophilis* followed *L. bulgaricus*, *L. helveticus*, and *L. casei*. Dyachenco et al. (1970) observed that 11 thermophilic lactobacillus strains were more proteolytic than three *S. thermophilus* strains tested. However, these investigations of casein hydrolysis by whole cells and others using cell extracts (van der Zant and Nelson, 1953; Brandsaeter and Nelson, 1956a,b; Poznanski et al., 1965; Tourneur, 1970) from lactic bacteria suggest that ranking of proteolytic activity, as reported above, may be dangerous. Often differences in proteolytic activity between strains within species may be greater than between species. Moreover, ranking of proteolysis measured in milk cultures may not be similar in cheese systems. For example, in a study of the proteolytic activity of rennet on casein, the water activity in the test system was critical. In cheese systems, β-casein hydrolysis was limited, whereas degradation of α-caseins was much more extensive. In dilute aqueous solution, α_{S1}- and β-casein were degraded at comparable rates (Creamer, 1971; Phelan et al., 1973). Similar conditions may also influence bacterial proteinase activity. This is visualized by researchers trying to relate the proteolytic activity of lactobacilli to the degree of cheese ripening. Although in test systems the lactobacilli showed extensive hydrolysis of casein, no effect on cheese ripening was found for *L. bulgaricus*, *L. lactis*, *L. helveticus*, *L. acidophilus*, *L. casei*, *L. fermenti*, *L. plantarum* or *L. brevis* when they were supplemented with starter cultures (Tittsler et al., 1948). Similar observations were made by Payens (1958) and Stadhouders (1968).

However, cell extracts of *L. bulgaricus* and *L. helveticus* hydrolyze α_s-casein most extensively, while β-casein was more resistant to hydrolysis in artificial systems (Ohmiya and Sato, 1969b). Cell extracts of *S. lactis* and *S. cremoris* were proteolytic on casein and casein fractions as measured by the increase of NPN, calcium-soluble moiety, free tyrosine, and free sialic acid (Ohmiya and Sato, 1967, 1968, 1969a, 1970b). In a recent study of the effect of *L. helveticus* and *S. cremoris* extracts in curd ripening, it was supposed that both organisms had a significant effect on casein hydrolysis and that the bacterial proteinases contributed more to the ripening of cheese curd than rennin (Ohmiya and Sato, 1972). Similar conclusions are reported by Kikuchi and Takafuji (1970).

Brevibacterium linens, the aerobic organism developing on the surface of some cheeses (Limburger, Trappist, Port du Salut), possesses high protease activity (Thomasow, 1950; Friedman et al., 1953; Tokita and Hosono, 1972). In Limburger cheese which is ripened by *B. linens*, a decreasing amino acid gradient was found from the surface to the center of the cheese. The same relative decreasing amounts of glutamic acid, leucine and isoleucine, valine, methionine, alanine, and tyrosine were found (Tuckey and Sahasrabudhe, 1957). The extracellular proteinase shows a degree of specificity for milk proteins. Besides casein, the proteinase only attacked seminal proteins and not bovine serum albumin, γ-globulin, β-lactoglobulin, or α-lactalbumin. Of the caseins, centrifuged casein, acid precipitated casein, and β-casein were hydrolyzed in decreasing order (Friedman et al., 1953). A 2% heated hemoglobin solution was hydrolyzed, but unheated hemoglobin or egg albumin was only slightly degraded (Tokita and Hosono, 1972).

Polypeptides were claimed to be the major product of proteinase activity. However, when whole cells lyse, a peptidase and a polypeptidase are released from the cell interior (Wong and Cone, 1964).

Cells of *B. linens* are thereby able to produce amino acids in cheese (Ades and Cone, 1969). Some types of yeast (Trichosporon and Debaromyces) also possessing proteolytic activity develop in the smear of surface-ripened cheese (Maginnis and Cone, 1958; Szumski and Cone, 1962).

The most extensive increase in water-soluble nitrogen in *B. linens* ripened cheese was found after 30 days of curing (Tokita and Hosono, 1972). Foissy (1973) investigated the influence of incubation time and growth media on the proteolytic activities from *B. linens* strains. Among all strains tested, a strain specific enzyme pattern was obtained.

Very few reports have been published on the nitrogen metabolism of propionibacteria. Virtanen and Kreula (1948) found that amino acids and especially proline were present in Swiss-type cheese. Cultures of propionibacteria grown on milk at 30 °C produced only small amounts of soluble nitrogen even over long periods of time (Virtanen, 1923; Werkman and Kendall, 1931; Klimovskii et al., 1965).

Proteinase activity of propionibacteria has not been detected according to reports by Werkman and Kendall (1931), Berger et al. (1938), and Langsrud (1974). However, Searles et al. (1970) assayed protease activity by whole cells

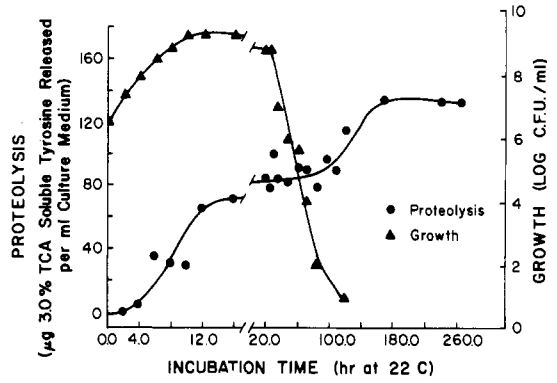

Figure 1. Growth and proteolysis of nonfat milk cultures of *S. lactis* C2 incubated at 22 °C without pH control: (•) proteolysis; (Δ) growth.

on casein and found specific protease activity twice as high for the propionibacteria strain as for five streptococcal strains. On the other hand, peptide hydrolyzing enzymes are found having a pH optimum of 5.5–6.0. These enzymes may be of considerable importance in ripening of Swiss-type cheese (Berger et al., 1938). Casein has a high proline content. The amount of proline released is especially high in cheese ripened by propionibacteria, and this has been connected with the sweet nutty flavor of these cheeses. Langsrud (1974) found a proline imidopeptidase and a proline iminopeptidase in *P. shermanii* which produced large amounts of proline from hydrolyzed casein (trypticase). Evidence revealed that the amount of these peptidases increased coincidentally with cell lysis.

RESULTS AND DISCUSSION

From the foregoing it can be assumed that starter organisms play a complex and subtle role in the proteolysis and thereby flavor development in cheese. The total proteolytic activity of the microbial cells including the extracellular, surface-bound, and intracellular enzymes is an important contributor to cheese proteolysis. However, the intracellular enzymes released by autolysis upon death of starter organisms (Ohmiya and Sato, 1970a,b; Law et al., 1974) may have a more direct role in flavor development. Intracellular exopeptidases could play a key intermediate link in the production of typical and/or atypical flavored cheese products. The importance of these exopeptidases would depend on their activity on peptide substrates previously formed by the action of rennet and endopeptidases present in the system.

This study was undertaken to evaluate the intracellular location and activity of the proteolytic enzyme system of some lactic acid bacteria under various conditions. Furthermore, the activity of these intracellular enzymes on protein breakdown products from partially ripened cheese curd was studied.

The following organisms were used in the experiments: *S. lactis* C2, *S. cremoris* ML1. Experimental conditions for growth, cell extract preparation, and enzyme assays have been previously reported (Schmidt, 1974).

Proteolysis of Growth Media. Figure 1 illustrates the growth and proteolytic activity of *S. lactis* C2 in reconstituted nonfat dry milk at 22 °C without pH control. Proteolysis occurred most rapidly during logarithmic growth phase in the first 12 h of incubation. As the stationary growth phase was reached, the proteolysis also stabilized. However, when the culture was entering the death phase, an appreciable increase in proteolysis was observed. Incubation beyond 180 h did not result in more than a slight increase in the extent of proteolysis. This pattern of proteolysis is indicating the function of an extracellular or surface-bound protease during logarithmic phase and the later more gradual burst of proteolysis could be due to the activity of intracellular enzymes released upon death and autolysis.

Table I. Proteinase Activity of Whole Cells and Cell Free Extracts of *S. lactis* C2 Grown in Casein Broth at 32 °C for 12 h

Cell preparation	Microbial growth[a]	Proteinase act.[b]	Protein concn[c]	Sp act.[d]
Cell extract				
A	2.40	136.67	6.72	20.34
B	1.58	286.67	16.53	17.34
C	1.10	153.33	8.99	17.06
Whole cells	2.40	323.33		

[a] Expressed as colony forming units (CFU)/ml of culture media × 10^9. [b] Expressed as the increase in μg of 10% Cl_3CCOOH soluble tyrosine released ml^{-1} of 0.1% casein substrate h^{-1} at pH 7.0 and 37 °C ml^{-1} cell. [c] Expressed as mg of protein/ml of cell extract. [d] Expressed as activity/mg of protein in cell free extract.

Proteolysis by Disintegrated Cell Extracts. Cell extracts were prepared by rupturing cell walls in the Eaton Cell and centrifugation (1200*g* for 15 min). The proteinase activities of three cell extracts (supernatant of ruptured cells, centrifuged at 12 000*g* for 15 min) and a whole cell suspension of *S. lactis* C2, as measured by the release of tyrosine equivalents from casein, are given in Table I. Extract A was from cells grown with pH 7.0 maintained during growth, while the pH of growth for extracts B and C was maintained at 6.0.

The specific activity was slightly higher for extract A. The whole cell suspension of *S. lactis* C2 also possessed proteinase activity indicating the presence of a membrane or surface-bound enzyme system as previously reported for other strains of *S. lactis* (Cowman et al., 1967; Cowman and Speck, 1967; Thomas et al., 1974; Kikuchi et al., 1974; Pearce et al., 1974). The activity of the proteinase from cell extracts was previously found to be highest at pH 7.0 and 37 °C (Amundstad, 1950; Baribo and Foster, 1952; Cowman and Speck, 1967; Krishna and Dutta, 1974).

Cellular Location of Proteinases. Ruptured cell preparations were fractionated by differential centrifugation as described in Figure 2. Soluble fractions of the disrupted cells of *S. lactis* C2 and *S. cremoris* ML1 grown at pH 5.2 were tested for proteolytic activity by measuring the increase in 10% Cl_3CCOOH-soluble tyrosine using casein as substrate. Specific activities of 5.20 and 3.30 (μg of 10% Cl_3CCOOH-soluble tyrosine released ml^{-1} of 0.1% casein $3\ h^{-1}\ mg^{-1}$ of protein at 37 °C, pH 7.0) were respectively recorded.

No detectable activity was present in the "particulate" or "ribosome" associated fractions. The fractions containing cell debris and whole cells were not tested.

Our "particulate" associated fraction was assumed to be membranous in nature on the basis of centrifugation procedure. Thomas et al. (1974) did not detect proteolytic activity in the membrane fraction from *S. lactis* 10 cells either.

An estimation by thin-layer chromatography of the dipeptidase activity of the "soluble" fraction of *S. lactis* C2 was conducted using several dipeptides found by previous authors to be reactive (van der Zant and Nelson, 1954; Cowman et al., 1968; Sorhaug and Solberg, 1973). Table II compares the reported results. There appears to be a variation in specificity of enzymes obtained from the four different strains of *S. lactis*. In general, leucyl and alanyl peptides were highly reactive with soluble extracts

Table II. Qualitative Estimation of Hydrolysis of Dipeptide Substrates by the Soluble Fraction of *S. lactis* C2 Grown in Lactic Broth at 32 °C, pH 5.2, Compared with the Activity of Cell Free Extracts from Other Strains Reported in the Literature[e]

Substrate	Dipeptidase act. for strain			
	C2[a]	I[b]	26[c]	3[d]
DL-Leucylglycine	+++	+++	++	0
DL-Leucyl-DL-alanine	+++	++	−	−
DL-Leucyl-DL-phenylalanine	+++	−	−	−
DL-Alanylglycine	+++	−	+++	+++
DL-Alanyl-DL-phenylalanine	++	+++	−	+++
Glycyl-DL-leucine	+	+	+++	−
Glycyl-DL-phenylalanine	+	++	−	0
Glycyl-L-tyrosine	+	0	+++	0

[a] Activity by thin-layer chromatography; incubation: 1 h at 37 °C. [b] Sorhaug and Solberg (1973); soluble fraction of *S. lactis* I; activity by electrophoretic technique; incubation: 6 h at 37 °C. [c] van der Zant and Nelson (1954); soluble fraction of *S. lactis* 26; activity by titration; incubation: 1 h at 37.5 °C. [d] Cowman et al. (1968); purified "intracellular proteinase" of *S. lactis* 3 activity by descending paper chromatography; incubation: 24 h at 37 °C. [e] 0, no evidence of hydrolysis; +, slight evidence of hydrolysis; ++, moderate evidence of hydrolysis; +++, strong evidence of hydrolysis; −, not determined.

Table III. Dipeptidase Activity of Cell Fractions from *S. lactis* C2 Grown in Lactic Broth at pH 5.2 and 32 °C

Peptides	Specific act.[a]		
	Soluble fraction	Ribosome associated fraction	Particulate associated fraction
Alanylglycine	5.19	0.60	0.68
Leucylglycine	2.87	0.43	0.85
Leucylalanine	3.11	0.33	0.85
Prolylglycine	0.34	0.10	0.09

[a] μmol of dipeptide hydrolyzed min^{-1} mg^{-1} of protein at 37 °C and pH 8.0.

from *S. lactis* C2, but glycyl peptides were poorly hydrolyzed. In contrast, *S. lactis* 26 (van der Zant and Nelson, 1954) was highly reactive with glycyl peptide substrates.

The soluble dipeptidase (*S. lactis* C2) was found to be saturated with substrate at a leucylglycine concentration of 30 mM. Maximum activity was observed at pH 8.0. A secondary pH optimum, however, was found between 6.25 and 6.50. This discontinuous pH curve could result from the presence of several enzymes in the crude soluble preparation. Optimum temperature for the soluble dipeptidases ranged from 45 to 50 °C. Of several substrates tested, alanylglycine gave the highest specific activity, followed by leucylalanine and leucylglycine.

The "particulate" and "ribosome" associated fractions had maximum activity at pH 8.0 and 45–55 °C. Table III compares specific activities of the different cell fractions toward several dipeptides. Obviously the soluble fraction was higher in activity than the other two fractions.

Influence of Nitrogen Source for Growth on Activity and Cellular Location of the Proteolytic Enzymes. The effect of whole casein in the growth media on proteolytic enzyme activity was studied. The "soluble" extract from cells grown in casein broth was only slightly higher in proteinase activity (sp act. 6.14) than that of cells grown in lactic broth containing tryptone (sp act. 5.20). The "soluble" dipeptidase activity measured with alanylglycine, leucylglycine, and prolylglycine as substrates

Table IV. Activity and Location of Peptidase Enzymes of *S. lactis* C2 Harvested in Late Log Phase of Growth from Casein Broth and Lactic Broth Incubated at 32 °C and pH 5.2

Cell location	Dipeptidase enzyme	Sp act.[a] when cells were grown in	
		Lactic broth	Casein broth
Soluble	Alanylglycinase	5.19	6.13
	Leucylglycinase	2.87	3.42
	Prolylglycinase	0.34	0.37
Particulate associated	Alanylglycinase	0.67	0.43
	Leucylglycinase	0.85	0.45
	Prolylglycinase	0.09	0.02
Ribosome associated	Alanylglycinase	0.61	0.09
	Leucylglycinase	0.43	0.22
	Prolylglycinase	0.10	0.04

[a] μmol of dipeptide hydrolyzed min^{-1} mg^{-1} of protein at pH 8.0 and 37 °C (average of duplicate trials).

Table V. Comparison of Dipeptidase Location and Activity of Different Strains of Lactic Streptococci Harvested in Late Log Phase of Growth from Lactic Broth at 32 °C and pH 5.2

Cell location	Peptidase	Specific act.[a]			
		S. lactis C2		*S. cremoris* ML1	
		pH 8.0	pH 5.0	pH 8.0	pH 5.0
Soluble	Alanylglycinase	5.19	0.52	3.67	0.21
	Leucylglycinase	2.87	0.38	2.66	0.19
	Prolylglycinase	0.34	0.00	0.30	0.01
Particulate associated	Alanylglycinase	0.68	0.19	0.59	0.05
	Leucylglycinase	0.85	0.08	0.30	0.04
	Prolylglycinase	0.09	0.00	0.03	0.00
Ribosome associated	Alanylglycinase	0.60	0.14	0.55	0.04
	Leucylglycinase	0.43	0.14	0.30	0.04
	Prolylglycinase	0.10	0.00	0.00	0.00

[a] μmol of dipeptide hydrolyzed min^{-1} mg^{-1} of protein at 37 °C and pH 8.0 or pH 5.0. (Average of duplicate trials.)

followed a similar trend (Table IV). However, cells grown in casein broth were lower in "particulate" and "ribosome" associated dipeptidase activity suggesting that the presence of tryptone (partially hydrolyzed casein) in the growth media induced these peptidase enzymes (Garvie and Mabbit, 1956; Westhoff and Cowman, 1970).

Strain Differences in Cellular Location. A comparison of location and activity of proteolytic enzymes of a "bitter" (*S. lactis* C2) and a "nonbitter" (*S. cremoris* ML1) producing strain was conducted. Cells of *S. lactis* C2 possessed slightly higher "soluble" proteinase and alanylglycinase, and "particulate" and "ribosome" associated dipeptidase activity than did cells of *S. cremoris* ML1 (Table V). The two strains were similar in "soluble" leucylglycinase and prolylglycinase activity.

When the dipeptidases were assayed at pH 5.0 instead of pH 8.0, the *S. cremoris* strain had generally lower dipeptidase activity in all cellular fractions than did *S. lactis* C2 (Table V).

These findings suggest that the complex phenomenon of bitter peptide removal by nonbitter strains cannot simply be related to the activity of one specific enzyme like proline iminopeptidase (Sullivan et al., 1971). Sullivan and Jago (1972) hypothesized that the most important factor in development of bitterness is the product of total proteinase activity of the cell and the starter population. The data referred to in Table V suggest that this hypothesis can be acceptable.

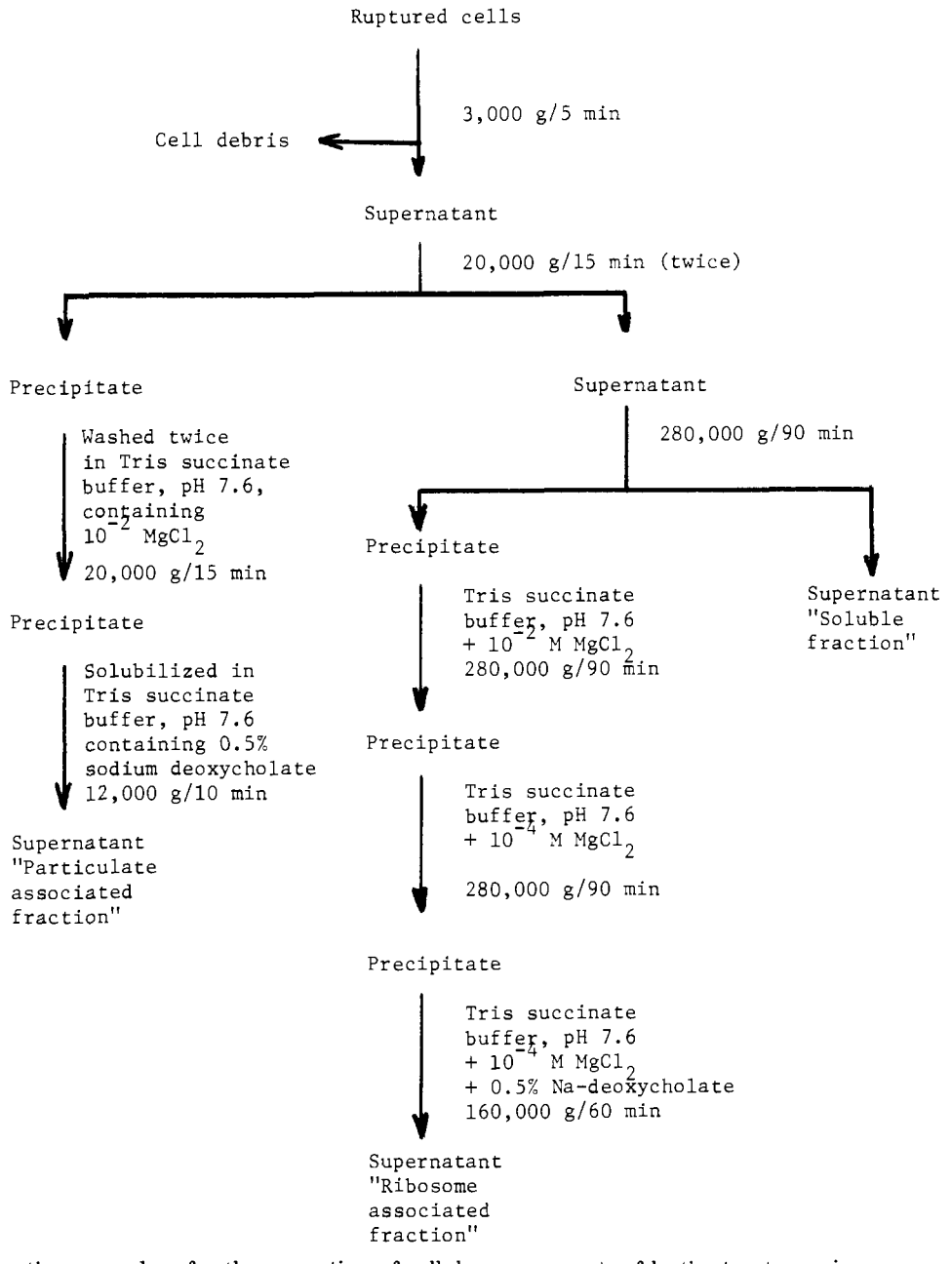

Figure 2. Fractionation procedure for the separation of cellular components of lactic streptococci.

Influence of Growth Phase on Proteolytic Activity. The influence of growth phase on lactic streptococcal proteolytic enzyme activity was studied. We tried to determine if the type and quantity of enzymes present during later growth phases can play a role in cheese proteolysis. The intracellular proteinase activity of *S. lactis* C2 was 10% less in late stationary phase relative to the value in late logarithmic phase. In early death phase, the reduction in activity was about 80%. The "soluble" proteinase of *S. cremoris* ML1 was much more affected. At late stationary phase, the activity was reduced by 80%.

Soluble and particulate associated alanylglycinase activity of *S. lactis* C2 remained fairly constant throughout the growth curve (Table VI). The ribosome associated alanylglycinase, however, was reduced about 70% in cells harvested in early death phase. Like the alanylglycinase, the leucylglycinase activity followed the same pattern throughout the growth phases.

Soluble prolylglycinase of C2 increased slightly by early death phase while the particulate associated activity decreased slightly. The ribosome associated prolylglycinase was drastically reduced by early death phase.

The soluble alanylglycinase activity of *S. cremoris* ML1 is more affected by growth phase than that of *S. lactis* C2,

Table VI. Effect of Growth Phase on Alanylglycinase Activity in Cell Fractions of *S. lactis* C2 and *S. cremoris* ML1 Grown at 32 °C and pH 5.2

		S. lactis C2		*S. cremoris* ML1	
Cell location	Growth phase	Sp act.[a]	% of log	Sp act.[a]	% of log
Soluble	Late log	5.19	100.0	3.69	100.0
	Late stationary	5.16	99.3	1.16	31.5
	Early death	4.97	95.8		
Particulate associated	Late log	0.68	100.0	0.59	100.0
	Late stationary			0.22	36.8
	Early death	0.70	103.2		
Ribosome associated	Late log	0.60	100.0	0.55	100.0
	Late stationary			0.18	32.4
	Early death	0.19	31.3		

[a] μmol of alanylglycine hydrolyzed min^{-1} mg^{-1} of protein at 37 °C and pH 8.0 (average of duplicate trials).

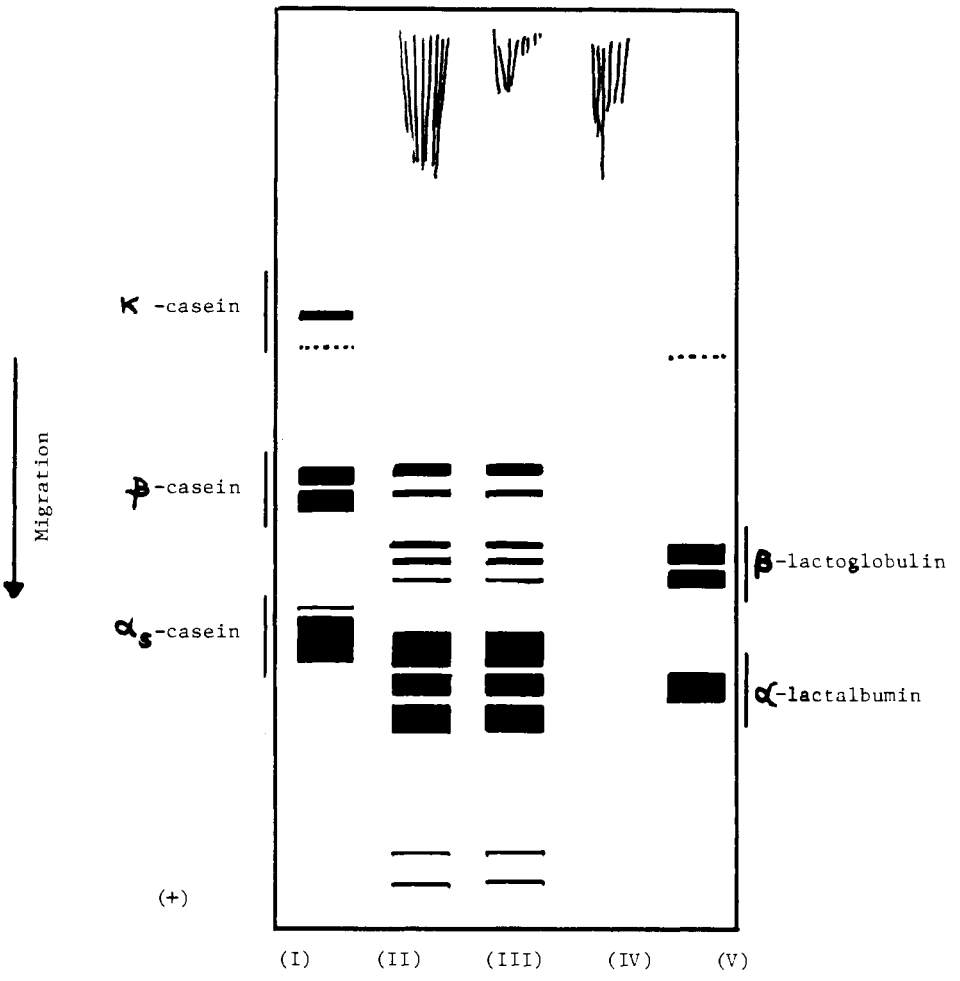

Figure 3. Urea starch vertical gel electrophoretogram of fractions obtained from rennet curd: (I) isoelectric casein standard; (II) curd solution; (III) fraction A from hollow fiber separation (mol wt 30 000); (IV) fraction B from hollow fiber separation (mol wt 5000-30 000); (V) isoelectric whey standard.

Table VII. Ratio of Alanyl Peptidase to Prolyl Peptidase Activity of Cells of *S. lactis* C2 and *S. cremoris* ML1 with Growth Phase of Harvesting Cells

Lactic streptococci	Growth phase	Ratio of specific act., AGase/PGase
S. lactis C2	Late log	15.3
	Early log	10.6
S. cremoris ML1	Late log	12.2
	Late stationary	6.0

and was found to be substantially reduced at all cellular locations in cells harvested during late stationary growth phase. Soluble leucylglycinase appeared to be slightly less affected by growth phase than alanylglycinase. The activity of particulate and ribosome associated leucylglycinase was, as the alanylglycinase, considerably decreased in cells harvested in the late stationary phase of growth. The soluble prolylglycinase of *S. cremoris* ML1 retained more activity (64%) than the alanyl- (32%) and leucyl- (51%) glycinase in the late stationary phase.

Data shown in Table VI suggest, in general, that the intracellular peptidase system possesses sufficiently high activity during the later phases of growth to play a role in cheese ripening following cell lysis. Perhaps a more meaningful comparison for assessing potential formation and removal of bitter peptides by lactic streptococci would be the ratio of general (alanylglycinase) dipeptidase to proline imino peptidase activity (Table VII). The alanylglycinase/prolylglycinase ratio of the bitter strain (C2) was slightly higher than the nonbitter strain (ML1) during the late logarithmic growth phase and was considerably higher during later growth phases.

Table VIII. Distribution of Nitrogen between Fractions Obtained by Hollow Fiber Fractionation of Rennet Curds

Curd	Fraction[a]	Nitrogen, mg/ml	Fraction vol, ml	Total nitrogen, mg	% of total
Curd 1	A	13.34	100	1334	81
1 ml of rennet/ 5 l. of milk	B	1.99	100	199	12
34 days at 22 °C	C	1.21	100	121	7
Curd 2	A	4.53	20	91	30
2.5 ml of rennet/ 5 l. of milk	B	1.36	35	48	15
45 days at 32 °C	C	2.10	80	166	55

[a] Molecular weight range of fraction based on nominal molecular weight cut off the hollow fiber used. A = >30 000, B = 5000-30 000, and C = <5000.

Rennet Curd Investigations. Rennet curd prepared without starter organisms was fractionated with the intent of studying the effect of prior proteolysis on the "soluble" enzymes of lactic streptococci. Proteolysis of the rennet curd was enhanced by increased rennet concentration, higher ripening temperature, and longer ripening time.

Solubilized curds were fractionated into components with molecular weights greater than 30 000 (A), from 5000

Table IX. Proteolysis of the Lactic Streptococcal Soluble Extract on the >30 000 Molecular Weight Fractions Obtained from Rennet Curd and a Whole Casein Substrate

Substrate	Specific act.[a] *S. lactis* C2	Specific act.[a] *S. cremoris* ML1
Whole casein	5.20	3.29
Curd 1	7.13	2.65
Curd 2	9.05	6.41

[a] μg of Cl_3CCOOH soluble tyrosine released ml^{-1} of 0.1% substrate mg^{-1} of protein 3 h^{-1} at 37 °C and pH 7.0 (average of duplicate trials).

to 30 000 (B), and below 5000 (C), by hollow fiber separation. The curd with lowest measurable proteolysis (Table VIII) had 80% of its components in fraction A and approximately 7% in fraction C. The curd with the highest measurable proteolysis had figures of 30 and 55%, respectively.

The 2% Cl_3CCOOH soluble nitrogen in fraction B could be correlated to the degree of proteolysis in the original curd. The 2% Cl_3CCOOH soluble nitrogen increased in fraction B in more proteolyzed curds, suggesting lower average size of the protein fragments. This trend was continuous with the increase in nonprotein nitrogen content of fraction C.

The average size of fractions A and B estimated by the ninhydrin ratio correlated to the degree of proteolysis in the rennet curd, with the lowest ratio observed in the fractions from curds of high proteolysis. The ninhydrin ratio determined in fraction C could not be correlated to the extent of proteolysis in the curds. Perhaps the presence of a high content of amino nitrogen in this fraction impaired comparison by the ninhydrin ratio technique.

Figure 3 shows a urea starch electrophoretogram of curd solution and of fractions A and B from hollow fiber fractionation. Proteolysis was apparent in the curd solution and fraction A by the number of minor zones and by the diffuse spreading nature of the zones.

Apparently the components of fraction B were not detectable by the electrophoretic technique used since electrophoretograms of the curd solution and fraction A were almost identical.

The major casein fraction which appeared to be most extensively hydrolyzed in the rennet curd was β-casein, as evidenced by the partial loss of this zone on the electrophoretogram of the curd solution. The α_s-casein fraction apparently was only partially hydrolyzed in the rennet curd. Zone 5 (counted from the lower part of the figure) of the curd was quite prominent, but slightly more diffuse in nature than the corresponding α_s-casein of the casein standard.

Degradation of Rennet Curd Fractions by Cell Extracts from Lactic Streptococci. Activity of the soluble extract of lactic streptococci on the rennet curd fractions was generally low compared to its activity on pure dipeptides. An apparent stimulation of activity by prior rennet action was suggested. This stimulatory effect could only be observed in fraction A (mol wt >30 000).

The activity of the soluble extract of C2 was consistently higher than that of ML1. Furthermore, the activity of C2 enzymes on rennet curd fractions was higher than on whole casein (Table IX). This was consistent with the enzyme system of ML1 except that the activity on the fraction from curd 1 was slightly (20%) less than the activity on the whole casein. Activity on this fraction by ML1 enzymes measured by amino acid release was also quite low. Reasons for this are not readily apparent.

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Effect of Microorganisms on Meat Proteins at Low Temperatures

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Most of the information on the titled subject has come from studies on the spoilage of meats rather than from studies in which specific proteins were subjected to the activities of microorganisms. The ecologic parameters of low-temperature fresh meat spoilage are such that the spoilage organisms consume nonprotein nitrogenous constituents and the simpler proteins preferential to those of the myofibrillar type. The predominant spoilage organisms under these conditions consist of several genera of gram-negative bacteria and there is no evidence of protein breakdown by this flora at the time of incipient spoilage. Myofibrillar proteins such as tropomyosin, α-actinin, troponin T, and actomyosin are attacked by the psychrophilic spoilers only after frank spoilage has occurred following prolonged storage. Prior to this point, the most dramatic change brought about by the spoilers is an increase in hydration capacity of meat proteins. Because the increased hydration capacity is related to increases in amino sugars and amino sugar complexes of bacterial origin and because these compounds possess the inherent capacity to increase protein hydration, it is postulated that they are necessary precursors to the ultimate activities of bacterial proteases and possibly meat cathepsins.

A careful search of the literature reveals only a few studies in which the effect of microorganisms on meat constituents was sought. Most of what is known about the effect of the spoilage flora on meat muscle proteins has come from studies on the effect of the spoilage flora on intact meats. Our present knowledge of the effect of microbes on meats is, therefore, limited mainly to the information which can be obtained by this general approach. The research of microbiologists on meats during the past 80 years has been prompted by concerns for the detection and prevention of microbial spoilage along with interests in meat preservation and the possible toxic effects of consuming spoiled meats. These aspects have been reviewed elsewhere (Ingram and Dainty, 1971; Jay, 1972). Studies on the mechanism of spoilage have received attention only recently.

This report is a summary review of our state of knowledge of the specific effects of microorganisms on meat proteins. Since most of this information is derived from studies on meat spoilage, it is desirable to view the meat spoilage process from the standpoint of the ecological parameters that affect the growth and activity of microorganisms in meats.

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CHEMICAL COMPOSITION OF MEATS

The approximate composition of meats such as beef and pork is presented in Table I. While the protein content ranges from 16 to 22% with an average of 18.5%, it should be noted that nonprotein nitrogenous substances constitute 1.5% and carbohydrates approximately 1.0%. When provided with complex and simple nutrient sources, microorganisms will invariably or always utilize the simpler constituents preferential to the more complex ones such as proteins. The protein-sparing actions of free amino acids, nucleotides, and related compounds in spoiling beef have been demonstrated (Jay and Kontou, 1967). Once the simple nitrogen sources have been exhausted, the simpler proteins such as those from the sarcoplasm are uitilized (Jay, 1966). Due to the generally low level of carbohydrates in meats, spoilage bacteria effect the deamination of amino acids and use the remaining molecules as energy sources with a consequent increase in NH_3 and H_2S in the spoiling meats. With the increase in NH_3, the usual postmortem beef pH of 5.6 to 5.8 begins to