1974

Proline production by propionibacteria

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by

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A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Food Technology
Major: Food Technology (Bacteriology)

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1974

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INTRODUCTION

Of the different varieties of ripened cheeses, Swiss ranks third in total production in the United States after Cheddar and Italian cheeses. Swiss or Emmentaler cheese is characterized by having an elastic body with smooth texture; uniform, smooth eyes; and a unique, nut-like, sweet flavor. These factors are so closely interrelated that poor texture and irregular eye distribution can arise from a poor body. Flavor may also depend, to a certain degree, on this relationship.

The name "Swiss cheese" implies that it was first produced in Switzerland. It is called Emmentaler cheese in Europe and originated in the Emme Valley, Canton of Bern, in Switzerland about the 15th century (41). Other spellings of Emmentaler may be found in the literature. In the United States, production of Swiss cheese originated in the 1860's in Ohio. This cheese originally was made in wheels with firm rinds, individual wheels weighing up to about 100 kg each; large amounts now are made in rindless blocks weighing about 36.4 to 40.9 kg (51). To facilitate cutting and packaging operations, some plants make larger blocks weighing about 91.0 kg.

Traditionally, Swiss cheese was made from raw milk, but in 1938, a method to make Swiss-type cheese from pasteurized milk was developed in Iowa (61). The method was closely

related to those used in Denmark, but has since been further modified (345). European countries other than Denmark also have developed Swiss-type cheeses. A variety closely related to Swiss cheese is Gruyére, originally produced in France since 1288 (36). This cheese is made in smaller wheels and undergoes surface ripening, therefore having a stronger flavor arising from the proteolytic activity of the surface-grown microorganisms. There are other cheeses whose distinctive characteristics depend on growth of propionibacteria. They all possess eyes of various sizes and have the related nut-like, sweet flavor (188).

High quality Swiss cheese is dependent on microbial fermentation of milk constituents. Propionic-acid bacteria are necessary to produce eyes and the typical flavor. Streptococcus lactis and Streptococcus cremoris are sometimes included for acid production, Streptococcus thermophilus to improve general quality, and a Lactobacillus species to control abnormal fermentation (67). Propionic-acid bacteria are the special microorganisms of Swiss cheese; their presence in large numbers is necessary to develop the characteristic flavor and eyes that distinguish Swiss cheese and related varieties from all other cheeses.

Traditionally, Swiss cheese flavor has been evaluated organoleptically. Chemical methods were usually not sensitive enough, and only the influence of compounds such as

propionic and acetic acid could be evaluated with some degree of accuracy. With the development of chromatographic methods for amino acids, fatty acids, and volatile compounds, chemical analysis of Swiss cheese flavor compounds became possible (82, 118).

Amino acids were quite early attributed importance for Swiss cheese flavor by some workers, while others regarded their influence with greater reservation. Virtanen and Kreula (241) found that proline, a sweet amino acid, was present in high amounts in Swiss cheese, and they indicated that proline might cause the sweet flavor so characteristic of Swiss cheese. A relationship between proline and propionic acid content and flavor was believed to exist, however, some workers could not confirm this belief.

Because of these differences in opinion over the importance of proline for Swiss cheese flavor, this investigation was undertaken. Also, we wished to determine if part of the proline in Swiss cheese is produced by propionic-acid bacteria. First, a study of commercial Swiss cheese was initiated to relate the amount of proline, other amino acids, groups of related amino acids, and the ratio of proline to propionic acid to Swiss cheese flavor. Then, we wished to determine if the propionic-acid bacteria could produce proline, by what mechanism(s) proline was produced, and if strain

differences could be observed. Influence of factors such as nitrogen sources, pH, and temperature on proline production also were to be determined. The relationship between cell autolysis, enzyme release, and proline production is of additional interest.

LITERATURE REVIEW

The manufacture of high-quality Swiss cheese is dependent on controlled microbial fermentation of milk constituents. Propionic-acid bacteria are necessary to produce the eyes and typical flavor of this cheese variety. Streptococcus lactis and Streptococcus cremoris are sometimes included for early acid formation, Streptococcus thermophilus to control general quality, and Lactobacillus species to prevent abnormal fermentations during the later stages of curd making.

This review will relate flavor production in Swiss cheese to microbial growth with special emphasis on nitrogen compounds and nitrogen metabolism.

For details of the manufacture of wheel or drum and rindless block Swiss cheese consult the monograph "Swiss Cheese Varieties" by Reinbold (188). For additional information on Swiss cheese flavor development and microbiology refer to the review by Langsrud and Reinbold (120, 121, 122, 123). For details of the general nutrition and metabolism of propionibacteria see the review by Hettinga and Reinbold (74, 75, 76).

Swiss Cheese Flavor

In the past it was believed that only one or a few compounds were responsible for Swiss cheese flavor. Propionic and acetic acid are produced in quantities generally paralleling

flavor development so Babel and Hammer (4) proposed that propionic acid was the most important contributor to Swiss cheese flavor. Later, Virtanen and Kreula (241) attributed the characteristic sweet flavor of Swiss cheese to its proline content. Hintz et al. (82) found that a minimum proline content of 2.0 mg per g and a minimum propionic acid content of 5.0 mg per g of cheese were necessary to produce typical Swiss cheese flavor. Later, Langler et al. (118) suggested that compounds such as acetaldehyde, methyl ketones, diacetyl, and dimethyl sulfide also were important for Swiss cheese flavor.

The earlier concept that cheese flavor is due only to a few compounds has now been replaced by the "component balance theory" of Kosikowski and Mocquot (112), which states that a relatively small number of compounds are responsible for the differences in flavor of different cheese varieties. The more important classes of chemical compounds thought/known to be associated with cheese flavor include certain fatty acids (158), aldehydes (90), methyl ketones (71), diacetyl (25), amines (209), peptides (72), sulfur compounds (248), and amino acids (241); other compounds such as esters (35), alcohols (56), partial glycerides (130), lactic acid (146), and salt probably also have some effect. Neutral fat, paracasein, and moisture cannot be ignored since the taste threshold concentrations of flavor compounds vary in fat and water solutions (158). These compounds are found in all types of

cheese to be responsible for a basic cheese flavor, but it is the amount and the proportion of a few specific compounds that produce the typical flavor of a certain cheese variety. Extreme shifts in proportions of these chemical compounds from the normal cheese lead to abnormal flavors. All components found in Swiss cheese may be considered important because of synergistic effects, even if they are found in lesser amounts than their threshold value. Other compounds present in amounts that are detected as off-flavors may still be essential for typical flavor when present in lower amounts (50). Generally, it may be said that the compounds mentioned in the beginning of this discussion are the important flavor compounds of Swiss cheese, but that the other compounds mentioned later in this discussion are necessary to give a balanced and full Swiss cheese flavor.

General Aspects of Swiss-cheese Ripening

After Swiss cheese curd has been removed from the press, brined, and prestored for 7 to 14 days, it is transferred to the warm room where the temperature is 21 to 25 C. During the period in the warm room, the cheese body becomes elastic and the principal ripening of the cheese, with active growth of propionibacteria, takes place forming the typical eyes of Swiss cheese (234). When the eyes are large enough, the

cheese is moved to the curing cellar or "finished cooler" and kept there at 2 to 5 C for a period of 2 to 9 months. A ripening period of at least 6 months should develop the fine, full flavor characteristic of a well-ripened Swiss cheese (51); however, in the United States today, most commercial Swiss cheese is much younger when sold (188).

During the time in the press, high-temperature lactic-acid bacteria rapidly hydrolyze lactose in the young cheese to glucose and galactose (184), which, by the Embden-Meyerhof pathway, is oxidized to lactic acid by the same bacteria (1). The lactic acid in the cheese is a readily assimilable substrate for the propionibacteria. Sherman (206) first reported propionibacteria to be essential for eye formation as well as production of the characteristic sweet flavor of Swiss cheese.

A large part of the lactic acid in Swiss cheese is produced by homofermentative lactic-acid fermentation, but a small portion also is produced chrough the heterofermentative pathway. Moderate amounts of acetic acid are found in the cheese curd after pressing (113). Because of this heterofermentative activity, carbon dioxide, which promotes growth of propionibacteria, also was found (70). Lactic-acid bacteria also reduced the redox potential of the Swiss cheese to -200 mv, which favors the growth of propionibacteria (98). Another important factor which favors growth of propioni-

bacteria is the pH-value of the cheese as it comes out of the press. The pH must be between pH 5.0 and pH 5.3 to produce good Swiss cheese. If the pH is lower than 5.0, growth of propionibacteria will not take place (235). The changes in pH and the redox potential, and also the carbon dioxide production are caused by the metabolic activities of the high-temperature starters.

During the warm room and curing room treatment of Swiss cheese, the numbers of S. thermophilus and Lactobacillus helveticus decline (38), even though it has been stated that mixed cultures of these two species may grow at temperatures as low as 15 C (83). The primary reason for this numerical decline is the elimination of lactose as an energy source and the formation of metabolic products in the cheese that may have an adverse effect on growth, especially as the cheeses are ripened near their minimum growth temperature. Lactobacillus casei, however, grows during ripening, and some investigators consider it an important ripening organism (38).

Growth of propionibacteria is favored in the warm room. Frazier and Wing (54) in 1931, stated that significant growth took place after about 3 weeks in the warm room, and during this time eye-formation begins. After 2 weeks in the warm room, the population of propionibacteria had increased 3 to 980 times the corresponding numbers present in curd in the press (117). This number was dependent on the initial pH of

the cheese out of the press. Maximum numbers of propionibacteria were found after 6 or 8 weeks when the initial pH had been between 5.15 and 5.37 when the cheese came out of the press. With initially lower pH values, maximum population occurred between 10 and 13 weeks. The population of propionibacteria showed a positive correlation with pH from the press. After the maximum number of propionibacteria was reached and sufficient eye formation had occurred, the cheese was transferred to the cold room. The number of propionibacteria slowly decreased during further ripening in the cold room (38, 117), even though some strains of Propionibacterium grow at temperatures as low as 7 C (157).

The distribution of propionibacteria in conventional Swiss cheese with rinds has been determined (189). Very few propionibacteria (1 x 10^6/g cheese) were found just below the surface of the cheese. Samples at 2.22 cm below the surface contained many more propionibacteria (74 to 1,200 x 10^6/g cheese) than the exterior portion, and the number progressively increased toward the center of the cheese (410 to 2,200 x 10^6/g cheese). Decrease of propionibacteria toward the exterior possibly results from such unfavorable conditions as lower moisture, higher salt concentrations, and higher redox potential. Rindless Swiss cheese has a more uniform distribution of propionibacteria because of the alleviating influence of the impermeable wrapper.

During curing, diverse chemical changes are brought about in Swiss cheese because of glycolysis, lipolysis, and proteolysis. Lactic acid is broken down to propionic and acetic acid, and proteolysis takes place. Flavor development depends on the relative amounts of lower fatty acids produced (119). Lower free fatty acids are related to the lactate content of the Swiss cheese, and lactate content is related to the lactose content in the cheese the day after manufacture. Cheese of good quality contains large amounts of propionic and acetic acid and small amounts of butyric and higher fatty acids, Swiss cheese flavor is strong and rancid (119). Sahli and Lehmann (193) showed that a well-ripened Swiss cheese contained 0.3% (by weight) acetic acid and 0.4% propionic acid but also, at least 0.012% butyric acid.

Proteolysis takes place during curing and the content of free amino acids in Swiss cheese increases. Free amino acids in amounts from 3.2 to 4.1 mg % were recognized as normal for Swiss cheese (68). Protein breakdown is delayed in Swiss cheese containing copper (94). In a study at Iowa State University on Iowa-style Swiss cheese, however, no retardation of proteolysis due to copper was observed, and, in most cases, higher proteolytic values were observed (Maurer and Reinbold, unpublished results). Propionibacteria do not affect proteolysis significantly (101). Most of the characteristic sweet flavor of Swiss cheese has been attributed by

some workers (241) to the proline content, which is higher in Swiss cheese than in any other cheese. Other workers (114) believe that proline and the ratio of propionic acid to proline is important for Swiss cheese flavor. Besides these two important compounds, many volatile carbonyl compounds in low concentrations have been isolated and these carbonyl compounds may be important for flavor (118).

The pH of Swiss cheese rises in the cold room, the warm room, and the curing room. Cheese out of the press has a pH from 5.1 to 5.3, and these values rise about 0.05 to 0.1 pH units in the cold room (51). The increase in pH in the warm room is much faster and the pH will be about 5.5 at the time the cheese is moved to the curing room (51). The pH after 2 to 3 months should not be less than 5.5 (240). If this pH is not reached, then normal ripening does not occur. Sahli and Lehmann (193) reported that high-quality Swiss cheese has a pH value between 5.64 and 5.70.

A high-quality Swiss cheese should contain eyes of proper size and form, and the eyes should be evenly distributed in the cheese. Normal eyes should have a diameter from 1.25 to 2.54 cm and should have 2.54 to 7.62 cm between them (67).

During ripening of Swiss cheese, the body gradually becomes soft, less tough, and less elastic. These changes probably reflect the solubilizing properties of proteolytic enzymes (51). Watson (249), however, observed that production

of a desirable soft texture in cheese was not necessarily coincident with the highest degree of proteolysis, quite the opposite actually being true. Variation in the colloidal structure of cheese, as shown by water binding, must be important in body characteristics. Swiss cheese tends to become more firm and less pliable as it loses moisture (102).

Body characteristics of Swiss cheese are due to the nature and state of the colloidal nitrogenous substances (103). Elasticity of cheese is highly temperature variable. Texture varies, not only from cheese to cheese, but also within an individual cheese (104, 105). Body characteristics of Swiss cheese were attributed by Koestler (106) to "hard nitrogenous substances" resulting from high cooking temperature, which gives the cheese, together with a relatively low moisture content, its characteristic properties. Physical properties of cheese are determined not only by chemical transformations occurring during lactic and propionic-acid fermentation, but also by the inner structural relation of the cheese curd. Characteristic for Swiss cheese is a high degree of elasticity, moderate firmness of the body, and high water-binding capacity. Mocquot et al. (145) found that total and plastic deformation of Swiss cheese was highly correlated with temperature and moisture. Eye number and size increased as total and plastic deformation increased. Rheological studies of Iowa-style Swiss cheese containing different amounts of copper showed

that copper content did not influence strength of the cheese but that an increase in copper content was highly correlated with an increase in elasticity and viscosity (Maurer and Reinbold, unpublished results).

Nitrogen Compounds

Considerable attention has been directed to the importance of amino acids in Swiss cheese. Some workers attribute major significance in flavor production in Swiss cheese to amino acids. Others regard the influence of amino acids on Swiss cheese flavor with greater reservation. Amino acids, doubtless, have an influence on cheese flavor, and a high glutamic acid content has been shown to have a direct influence in this regard (69). Even peptides seem to have taste-forming influences (222). Proteins, on the contrary, induce no flavor but are important for the body and texture of cheese.

Amino acids in Swiss cheese

Virtanen and Kreula (241) studied the relation of Swiss cheese flavor to the amino acids present in cheese and found that proline and hydroxyproline had a sweet taste and constituted a high proportion of the amino acids in the cheese serum. They, therefore, concluded that proline might be the cause of the sweet flavor. The high arginine content of casein also was thought to be another factor influencing flavor; however,

during ripening, 66% of the arginine was lost, and it was concluded that the disagreeable flavor of arginine had no effect (241). Two-thirds of the nitrogen compounds of tear fluid of Swiss cheese consisted of free amino acids and one-third of peptides and proteins. Proline was abundant in the tear fluid (242). Storgårds and Hietaranta (220) considered that a portion of the flavor was due to the higher amounts of glutamic acid and aspartic acid in Swiss cheese than in other hard, rennet cheeses. The amount of acidic amino acids increased and the amount of basic amino acids decreased during storage of Swiss cheese.

The first qualitative study of all amino acids in Swiss cheese was performed by Block (15) in 1951. All the amino acids present in casein, as well as different degradation products of amino acids, were found using paper chromatography. Storgårds and Lindqvist (221, 222) also studied the amino acids of cheese quantitatively. They determined that the amount of proline in Swiss cheese was higher than in any other cheese. The quantitative amounts of amino acids in Swiss cheese were determined by paper chromatography by Kosikowski and Dahlberg (111) and Hintz et al. (82), while Antila and Antila (3) and Ritter et al. (190) measured the amino acid content using more exact ion-exchange chromatographic systems. The amino acid content of Swiss cheese tear fluid was measured by Schormüller et al. (200). These

results are found in Table 1 and are compared with the amino acid content of Edam and Cheddar cheese.

Not all amino acids are present in various Swiss cheeses and amounts vary widely among cheeses (82). Proline showed the widest quantitative variation, from 0 to 5.8 mg per g cheese. Amounts of cysteic acid, taurine, proline, lysine, and histidine tended to increase during storage, but the glycine content tended to be constant. The concentrations of aspartic acid, threonine, serine, glutamic acid, tyrosine, phenylalanine, and tryptophan present could not be related to the age of the cheese. A relationship between proline and propionic acid content and flavor was believed to exist although Jäger (84) found no correlation between amino acids and flavor. The work of Antila and Antila (3) and Ritter et al. (190) showed that there were distinct differences between Finnish and Swiss Emmental. The proportion of glutamic acid to proline in Swiss Emmental was 2:1; in the Finnish cheese, it was 2:3. The amount of arginine was 40 times higher in Swiss Emmental. These differences may be due to different manufacturing processes and different strains of starters used.

The sweet taste attributed to proline in Swiss cheese also may be due to the group of sweet amino acids: glycine, alanine, proline, serine, and threonine, but the proline content is much higher than any of the other sweet amino

Table 1. Free amino acids in Swiss cheese compared with those in Gruyére, Edam, and Cheddar cheese (mg/100 g cheese)

Amino Acid	Free amino acids in cheese								
	Swiss cheese, domestic[a]				Emmental[b]				
	No flavor	Mild	Medium	Pronounced	3 month	6 month	Gruyére[b]	Edam[b]	Cheddar[b]
Aspartic acid	260	Trace	0	160	9.2	16.7	32.9	2.1	37.2
Threonine					42.0	68.9	78.1	14.5	90.0
Serine					32.0	54.9	44.6	7.1	67.9
Glutamic acid	87	210	330	180	134.6	268.1	296.4	35.2	273.4
Proline	30	350	300	530	134.4	253.5	281.7	15.4	54.4
Glycine	0	90	120	190	16.5	43.1	49.3	3.5	32.6
Alanine					36.4	56.8	59.8	6.9	53.8
Valine					86.9	156.2	181.8	16.7	146.9
Methionine					28.1	50.3	53.2	6.0	67.6
Isoleucine					52.2	105.1	118.9	4.8	101.4
Leucine					117.0	179.5	177.9	42.6	190.1
Tyrosine					4.3	28.6	32.1	8.9	29.7
Phenylalanine					73.0	127.9	136.7	29.1	132.7
γ-Amino butyric acid					10.0	26.1	30.8	1.2	37.0
Ornithine					74.5	113.3	99.3	22.4	140.0
Lysine	230	150	100	950	146.5	222.0	222.8	24.6	216.2
Tryptophan	72	Trace	54	64	4.5	2.2	12.0	-	6.6
Histidine	0	350	110	0	51.2	86.8	68.1	5.0	58.2
Arginine					3.5	1.9	2.1	13.1	7.8
Tyrosine + phenylalanine	190	390	540	290					
Threonine + serine	190	170	130	450					

[a]Hintz et al. (82).

[b]Antila and Antila (3).

acids. The ratio of bitter amino acids to sweet amino acids was 2.33 in poor-quality Swiss cheese, but in high-grade Swiss cheese, it was 1.93 to 2.05 (45). Schormüller et al. (200) found that the amino acid composition of the tear fluid of Swiss cheese was qualitatively, but not quantitatively, equal to that of casein. Proline content was about twice as great in the tear fluid.

Production of peptides and amino acids

Rennet coagulation of Swiss cheese consists of two reactions: 1. A primary reaction with liberation of non-protein nitrogen from the casein for curd production, which is a very fast reaction. 2. A secondary reaction that starts the proteolytic degradation of the caseins (198). This proteolysis of casein has been followed by electrophoretic studies. The degradation of casein proceeds in different ways according to cheese types as shown by Lindqvist and Storgårds (126). Hard cheeses such as Svecia and Swiss cheese had an "alpha-ripening", which means that the greatest changes took place around the alpha-casein peak, but cheeses, such as Port Salut and Camembert, showed a "beta-ripening", with the greatest changes around the beta-casein peak. Instances of nonspecific casein degradation, as in Tilsit and Limburg, also were found. During ripening of Svecia cheese, Lindqvist and Storgårds (127) found that the first stage

was decomposition of a component of the alpha-casein peak, probably kappa-casein. Approximately at the same time, degradation of the beta-casein took place. Alpha-casein is not attacked until after a longer period of ripening. The same changes were observed with Swiss cheese (168) but Dȳlanyan et al. (45) do not believe that this division of the ripening process into different categories is justified.

The degradation of caseins could be caused by rennet, by the natural proteinase of milk, and by microbial proteinases, but Stadhouders (217) found that the proteolytic activity of rennet and the milk enzyme was of only minor importance during cheese ripening and that the predominant enzymes were microbial proteinases. Poznańnski and Rymaszewski (180) studied the proteolytic activity of S. thermophilus and Lactobacillus bulgaricus in Edam cheese ripening. Addition of L. bulgaricus gave a satisfactory flavor, better than S. thermophilus; addition of Micrococcus caseolyticus gave a bitter cheese (180). Only the endoenzymes of L. bulgaricus acted appreciably on whole casein, and the endoenzymes from S. thermophilus and M. caseolyticus had greater effects on intermediate products from casein. The activity of bacterial enzymes was low compared with the activity of rennin, and the activity of combined enzymes was greater on alpha$_s$-casein and kappa-casein than on whole casein (179). The enzymes produced by

S. thermophilus and L. helveticus decomposed, to a varying extent, para-casein degradation products formed as a result of rennet hydrolysis (178). Proteinases of lactic-acid bacteria seem to play a particular role in flavor development. Rapp (187) observed that L. acidophilus showed the highest proteolytic activity followed by L. bulgaricus, L. helveticus, and L. casei, in that order. Dyachenko et al. (43) observed that 11 thermophilic Lactobacillus strains showed higher activity than 3 S. thermophilus strains. Lactobacillus helveticus showed the highest activity. Since enzymatic differences between strains within species can sometimes be greater than between representatives of different species, ratings of this nature are risky at best. Studies by Ohmiya and Sato (155) indicated that the extent of casein hydrolysis by intracellular proteases of S. cremoris and L. helveticus is similar to that of rennin. Aseptically harvested and washed cells of S. cremoris and L. helveticus were sandwiched between aseptic rennet curd. The curd was then coated with wax and incubated. Rapid autolysis of the cells was shown to take place (153). The nonprotein nitrogen content increased, even in the aseptic curd during ripening at 10 C, but the increase was smaller than in aseptic curd inoculated with S. cremoris and L. helveticus (154). This indicates that rennet is more active than Stadhouders (217) assumed. Alpha$_s$-casein was most easily degraded by the lactic-acid bacteria. On the

contrary, Dyachenko and Shidlovskaya (44) observed that L. bulgaricus, L. helveticus, S. cremoris, and S. thermophilus decomposed beta-casein more rapidly than alpha-casein. The methods used for measuring proteolytic activity also are important. Lactobacillus thermophilus showed much greater activity when assayed by measurement of tryptophan and tyrosine than by formol titration, although the reverse was true for S. thermophilus (151). Proteolytic enzymes have not been found in propionibacteria (12, 101).

The products of the proteolysis of caseins are peptides and amino acids. Schormüller and Belitz (199) showed that during the first 14 days of ripening of Harz cheese the amino acid content increased and then decreased to the 21st day. The amounts of peptides increased progressively during this period. In all cheeses investigated, a characteristic peptide typical for casein degradation was isolated. Using electrophoresis, Storgårds and Lindqvist (223) investigated the peptide distribution of Svecia, Herrgård, Swiss, Port Salut, Blue, and Camembert cheese and found that all had a characteristic peptide composition. By more detailed studies it may be possible to isolate the different peptides and obtain a more exact picture of the differences in proteolysis between different cheeses.

Peptides may produce a bitter flavor, which is considered a defect in Swiss cheese. But usually during ripening, the

peptide content decreases (223). Since many of these peptides are phosphopeptides, which are not attacked by peptidases, phosphatases must remove the phosphates before the peptide can be degraded to amino acids. Phosphatases have been found in cheese, but because of their low activity in ripening cheeses, they may be the limiting factor for peptide degradation (198).

Peptidases are found in many microorganisms, but they have not been studied as much as proteinases (198). Bipeptidases, amino peptidases, and carboxypeptidases were found in sour-milk cheese (204), which showed the same pH-maximum as peptidases of microbial origin. The peptidases in the cheese were quite similar to the enzymes found in yeasts, bacteria, and animal tissues (198). Peptidases also have been found in propionibacteria, and these enzymes have an optimum pH close to 5.5 to 6.0 (12), which may be of importance in Swiss cheese. Schormüller and Müller (201, 202) were especially interested in the enzymes prolinase and prolidase in sour-milk cheese because free proline has always been found in cheese. Neither prolinase nor prolidase are found in fresh curd, but they develop during ripening from the outside to the inside of the curd mass. Their highest activity in sour-milk cheese was found close to the rind, and the activity decreased toward the core. Prolidase activity also has been found in Swiss cheese (202).

The peptides formed during casein decomposition may produce a bitter taste in the cheese if certain microorganisms are deficient in the peptidases that participate in peptide degradation (198). Stadhouders (217) found that bitter cheese most frequently developed at pH 5.2. The starters that decompose bitter polypeptides may differ entirely in characteristics from the nondecomposing starters. This capability was specifically marked for some strains and it was not connected with the capability to liberate free amino acids.

The difference in capability to decompose the bitter peptides may be because a pyrrolidone carboxylic acid at the N-terminal end of a hydrophobic peptide derived from casein may cause bitterness in some instances (48, 224). Sullivan and Jago (224) suggested that removal of bitterness by nonbitter starters was due to the presence of a pyrrolidone carboxyl peptidase, a view that was opposed by Exterkate and Stadhouders (48), who found this enzyme in higher amounts in bitter than in nonbitter strains of S. cremoris.

The amino acids produced by proteolysis and peptide breakdown will be more or less changed by microorganisms in the cheese. Changes in the amino acid spectrum of milk produced during growth of two strains of S. thermophilus and four strains of Lactobacillus have been recorded by Grudzinskaya and Koroleva (64). Amino acids present in considerable amount in the sterile milk were glutamic acid, glycine,

threonine, alanine, tyrosine, methionine, and valine. Streptococci used almost all of these amino acids during their early stages of growth in milk. Growth of L. bulgaricus and L. acidophilus left the glutamic acid content almost unchanged, reduced that of threonine, and led to accumulation of histidine, arginine, tyrosine, phenylalanine, leucine, cysteine, methionine, and valine. Combinations of streptococci and lactobacilli intensified both the utilization of amino acids present and the formation of others. Kiuru et al. (99) observed that, during eye formation in Swiss cheese, the quantity of certain amino acids either is constant or declines. After 90 days, some amino acids increase greatly, but others decrease. Free aspartic acid disappears probably because of aspartase, which has been found in propionibacteria (243). After 45 days the arginine content also is close to zero. Alanine, valine, serine, tyrosine, aspartic acid, glutamic acid, arginine, cystine, and methionine are degraded by propionibacteria (2).

The most important degradation products of amino acids with reference to flavor are probably amines, which are produced by decarboxylation of amino acids. The following amines have been found in Swiss cheese: tyramine (110), histamine (237), gamma-aminobutyric acid and cadaverine (15), and putrescine (228). The hard cheeses seem generally to have low amounts of these amines (67). The amino acid de-

carboxylases occur only sporadically in lactobacilli (198), and Dacre (34) found only one strain of Lactobacillus with a high amount of tyrosine decarboxylase. Amino acid decarboxylase activity has not been reported for propionibacteria. The tyramine content found in cheese has been attributed by Kosikowsky and Dahlberg (110) to tyrosine decarboxylase produced by streptococci, especially S. faecalis. Other spoilage bacteria, such as coliforms, also may produce these compounds (198).

Serine and threonine may be decomposed to alpha-amino butyric acid and alpha-alanine in sour-milk cheese according to Schormüller (198). Arginine, which has a repulsive, bitter, sweet taste, was shown by Schormüller and Tänzler (203), to be decomposed via citrulline and ornithine to putrescine by arginine desimidase and is, therefore, eliminated as a flavor-diminishing amino acid. Transamination reactions also take place during the metabolism of amino acids.

Nitrogen Metabolism of Propionibacteria

Very few reports have been published about the nitrogen metabolism of propionibacteria, and mainly then in relation to nutritional requirements. Propionibacteria hydrolyze proteins very slowly. In taxonomic studies, Werkman and Kendall (250) found that propionibacteria do not solubilize

gelatin. Pure cultures of propionibacteria grown in milk at 37 C produced only a small increase in soluble nitrogen over an extended period (239). This may indicate only very slow proteolysis. By using peptones as a nitrogen source, the propionibacteria grow much faster and reach higher numbers than in milk. From these observations one cannot conclude that peptides are utilized, because peptones contain high amounts of free amino acids and ammonium salts. However, Berger et al. (12) found a tripeptidase in Propionibacterium pentosaceum. The optimum pH of this enzyme was around pH 5.5 to 6.0. By all appearances this indicates that propionibacteria have the apparatus to utilize peptides for growth.

The nutritional requirements of propionibacteria have generally been regarded as complex, but amino acids, although beneficial, are not essential. Although Tatum et al. (229, 230) first observed that propionibacteria are able to utilize ammonical nitrogen, it was Wood et al. (253) who first grew these organisms with success through numerous transfers in an ammonium sulfate medium. This shows that propionibacteria contain the necessary enzymatic apparatus for biosynthesis of all amino acids. But, even if amino acids are not essential for growth, their presence stimulates growth. The removal of some amino acids from an amino acid mixture in a medium slows down the growth rate. Fromageot and Chaix (55) reported that sulfur-containing amino acids are growth

stimulatory. Tatum et al. (229, 230) observed that glutamic and aspartic acid could replace ammonium nitrate as a growth factor. Antila (1) reported that when glutamic and aspartic acid were omitted from a total amino acid mixture a large reduction in growth resulted. Omission of ammonium chloride did not have that dramatic effect. Removal of all three constituents showed about the same result as removal of glutamic acid alone. This indicates that removal of these amino acids, which are branch points for biosynthesis of many important nitrogen compounds, limits the growth of propionibacteria.

Antila (2) observed that three strains of propionibacteria degraded alanine, valine, serine, tyrosine, aspartic acid, glutamic acid, arginine, cystine, and methionine. Propionibacterium petersonii was shown in experiments by Kiuru, to produce large amounts of ammonia from DL-alanine, L-aspartic acid, DL-serine, D-arginine, and L-cystine (1). Nitrates retarded the amino acid deamination activity of propionibacteria during cheese ripening (167). Virtanen and Tarnanen (243) reported that propionibacteria contain the enzyme aspartase which deaminates aspartic acid to fumaric acid and ammonia. The aspartase was purified by Ellfolk (47). This short summary shows that the nitrogen metabolism of these industrially important microorganisms has largely been neglected, and nothing is really known about how they effect

the production of nitrogen compounds and especially proline in Swiss cheese.

Proline Production

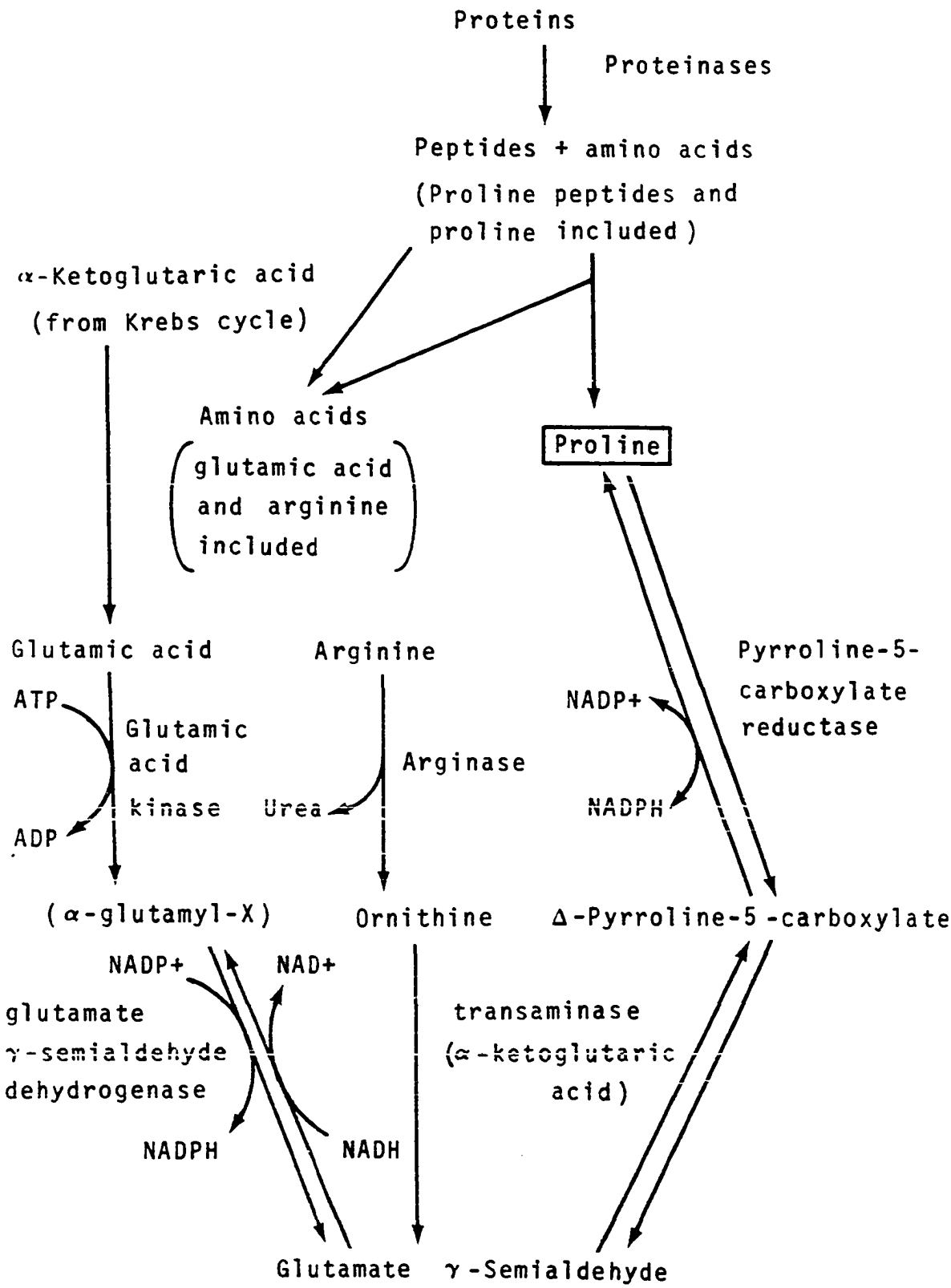
Proline can be produced microbiologically by three different methods: 1. Proteolysis, 2. Peptide hydrolysis, and 3. Proline biosynthesis as shown in Figure 1. These systems will be discussed generally, because very little research on these aspects of propionibacteria has been performed.

Bacterial proteases

Many bacterial proteases have been described in the literature. However, most of these are believed to be extracellular. The evidence for extracellular enzymes does not always satisfy the rigorous criteria to define the enzyme as extracellular as opposed to material released by autolysis (177). Still, even truly extracellular enzymes are synthesized in an intracellular environment, possibly in an inactive form, because otherwise they would be able to degrade cellular proteins. The protease is probably activated only when it reaches the cell wall (136). For more details about extracellular proteases refer to "The Enzymes, Vol. III" edited by P. D. Boyer (18).

In an earlier part concerning production of nitrogen compounds in cheese, the proteolytic enzymes of lactic and high

Figure 1. Possible pathways for proline production by microorganisms in Swiss cheese


Proteins
Proteinases
Peptides + amino acids
(Proline peptides and proline included)
α-Ketoglutaric acid
(from Krebs cycle)
Amino acids
(glutamic acid and arginine included)
Proline
Glutamic acid
Arginine
Pyrroline-5-carboxylate reductase
ATP
Glutamic acid kinase
ADP
Arginase
Urea
NADP+
NADPH
(α-glutamyl-X)
Ornithine
Δ-Pyrroline-5-carboxylate
NADP+
NAD+
glutamate γ-semialdehyde dehydrogenase
NADPH
NADH
transaminase (α-ketoglutaric acid)
Glutamate
γ-Semialdehyde

temperature starter bacteria were discussed. The presence of extracellular proteolytic activity was suggested by van der Zant and Nelson (260) and Sasaki and Nakae (197) who observed hydrolysis of milk proteins by cell-free spent medium. Similarly, Miller and Kandler (140) reported the presence of an extracellular protease in L. bulgaricus. In both cases, the sample for extracellular enzymes was removed after 24 h when the cells were in the stationary phase, where cell autolysis probably would have started. To show extracellular proteases, the sampling should have been done when the cells were in early or mid log phase. Ducastelle and Lenoir (42) however, observed no extracellular protease activity in streptococci and lactobacilli. Miller and Kandler (140) also observed that the fastest production of low-molecular weight compounds took place during the first 12 hr, which may indicate an extracellular protease.

Only in one case has a detailed study of proteases been made of lactic-acid starters. Cowman and Speck (31, 32) studied the proteinases of S. lactis. They found an intracellular protease system and a membrane protease. The membrane proteinase was very unstable at low temperatures. The presence of two proteinases may indicate that the protein breakdown during early growth stages was caused by membrane proteinase, while the soluble proteinase, which is more stable, will continue the proteolysis. Intracellular proteinase has

been found in L. bulgaricus, but since it also produces nitrogen compounds in early log phase, a membrane proteinase may be present. In propionibacteria, the chance for a membrane protease is very low, because increase in low-molecular weight nitrogen compounds takes place after incubation over a very long time (239). So, possibly, the important bacterial proteolytic enzymes from the standpoint of casein hydrolysis in cheese by starters are membrane-bound and stable intracellular proteases. Under certain growth conditions breakdown of intracellular stable proteins takes place. This breakdown must depend on the presence of intracellular proteases. Protein turnover was first described by Mandelstam (131) in Escherichia coli which was grown under conditions of carbon and nitrogen deficiency. Willets (251, 252) found several other growth factors producing the same effect. Under conditions where bacteria are deprived of elements essential to growth some enzymes are more quickly degraded than others. Two classes of proteins with different turnover rates have been found (148, 173). The first class is quickly hydrolyzed, whatever the growth phase; the second class shows a very slow turnover during the exponential growth phase, but the turnover rate increases in the stationary phase. Abnormal proteins synthesized by the cell, and products of the β-galactosidase operon carrying nonsense mutation or deletion are rapidly degraded by E. coli during the exponential growth phase (57,

58, 171, 176). Two classes of intracellular proteases have been shown *in vivo* (183), one operating in all cells which can degrade abnormal proteins and the second class which operates in starving cells.

The fact that the protein hydrolysis only takes place during starvation indicates that it is regulated. The regulation mechanism is unknown. It may be important to study their action on other intracellular enzymes, because this may be an important facet in studying cellular regulation mechanisms. In *E. coli* the following enzymes have been shown to be degraded into small molecular species exhibiting different catalytic activities than that of the original enzymes: leucyl-tRNA-ligase (191), methionyl-tRNA-ligase (26), polynucleotide phosphorylase (231), and DNA polymerase (100). The degradation of these enzymes may be important at the time of cell division. The protease synthesis may normally be repressed or it may exist in latent form. Thus, starvation may induce synthesis of proteinase or activation of latent enzyme, and also the enzyme may be activated by some unknown regulator close to cell division.

Peptidases

The proteinases of high temperature starter bacteria and the proteolytic ability of rennin will, in Swiss cheese, lead to production of oligopeptides, dipeptides, and free

amino acids which can be utilized by propionibacteria in the warm room. The peptides produced could be hydrolyzed outside the cell by extracellular peptidases and be transported into the cell. In such a system no peptide transport system would be necessary. However, extracellular peptidases have only been reported in a few instances for a few species of bacteria which also produce extracellular proteinases (66, 181, 182, 247). Extracellular peptidases may provide a more efficient use of nitrogen from large peptides. Wagner et al. (247) observed maximum production of the peptidase in stationary phase, but believed that the enzyme was extracellular because addition of chloramphenicol and/or oxytetracycline to the culture medium reduced the enzyme production immediately, without an immediate effect on the number of viable cells.

As an alternative, peptides could enter the bacterial cell where peptide hydrolysis would take place. Much evidence has been provided supporting this theory (159, 163, 165, 169). Even with bacteria which can utilize proteins as a source for amino acids, the evidence generally supports an intracellular location of the peptidases, in contrast to the extracellular proteases (165).

Both peptides and amino acids are transported into the cell. Several different permeases which are specific for groups of amino acids or for a single amino acid, for example

proline, are used for amino acid transport (88, 89, 91). A free α-amino or imino group and a free carboxyl group are requirements for active transport. Dipeptides and oligopeptides are transported by two different systems. Dipeptide transport occurs via a specific permease(s), and the requirement is a free C terminal end and a free α-amino group (159, 163, 165). Payne (160) later showed that methylation (protonation of the α-amino group) of a dipeptide did not interfere with transport but acylation stopped the transport. No specific transport system was found for prolyl peptides (161). Kessel and Lubin (92) demonstrated that glycylglycine transportation took place with an active system, which indicates that dipeptide transport is mediated by active transport. The oligopeptide permease system can transport peptides containing different numbers of amino acids (159). The oligopeptide requires an α-amino group for transport, but no requirement for a C-terminal carboxyl group. The oligopeptide permease can transport peptides containing no carboxyl end and also esterified terminal carboxyl group (159, 163). No peptides larger than a certain size may enter the bacteria, and the size seems to be related to the hydrodynamic volume of the peptide (164). No proof for active transport has been found for oligopeptides.

Bacteria can utilize a wide variety of peptides for amino acid production. Much work has been done with peptidases, but

the results are difficult to compare, and in most cases these enzymes have not been extensively purified. Some work has been done with peptidase activity in bacteria used in the dairy industry. Berger et al. (12) reported acidopeptidases in P. pentosaceum which split peptides at optimum pH-values of 5.5 to 6.0. These pH-values are well suited for Swiss cheese, and should indicate some enzyme activity. Van der Zant and Nelson (261) studied the peptidase activity of extracts of S. lactis. The activity was the same in a protein medium as in a control medium without protein. The enzymes were stable to storage at different pH-values. Use of different metals showed that Mn^{++} increased the hydrolysis of glycyl-L-leucine, and when DL-alanyl-glycine was used as substrate, Co^{++} increased the enzyme activity. The results indicate the presence of more than one peptidase. Studies of the dipeptidase activity of L. casei indicates also more than one enzyme (19). Sorhaug and Solberg (216) observed, by starch gel electrophoresis, that S. lactis contained at least four different dipeptidases. Four peptidases also were isolated from S. thermophilus by Rabier and Desmazeaud (185). These enzymes may be very important for cheese ripening.

Most of the work on peptidases, however, has been done on E. coli. Simmonds and Toye (213) examined the cleavage of hydrophobic peptides and advanced indirect evidence that four peptidases were present: one, which is relatively in-

sensitive to ethylenediaminetetraacetic acid (EDTA), is activated by Co^{++}, and catalyzes the hydrolysis of Gly-L-Leu and Gly-L-Phe; the other two are EDTA sensitive, but they differ in their response to Co^{++} and Mn^{++}; and the fourth is associated with the ribosomes. Sussman and Gilvarg (225) deliberately limited their experiment to lysine homopeptides (di, tri, tetra), and found four peptidases: a Co^{++}-dependent peptidase capable of splitting dilysine; an EDTA-sensitive peptidase specific for trilysine; an endopeptidase that can hydrolyze tetralysine, but not di- and trilysine; and a metal-independent peptidase which could cleave all three lysine peptides. Payne (162) observed in a study of dipeptidases in E. coli, that probably there was a single Co^{++}-activated enzyme which showed a broad specificity toward dipeptides, and several enzymes with lower activities and narrower substrate specificity which was activated by Mn^{++}. The discussion shows that there are a large number of different peptidases in bacteria, but how large is unknown.

A few of the peptidases from E. coli have been purified and characterized in some detail. One was a peptidase specific for β-aspartyl peptides which showed no metal requirements (65). An aminopeptidase isolated by Vogt (246) was most active against methionyl peptides, and was inhibited by EDTA, with restoration of activity with Mn^{++} or Mg^{++}. Dick et al. (39) purified a ribosomal bound basic aminopeptidase in a stable

form which showed a strong activity toward leucyl, methionyl, threonyl, and lysyl peptides. In a report in 1971, Matheson et al. (132) suggested that more than one basic aminopeptidase may be present on the ribosome. Brown (22) reported the isolation of dipeptidase M from E. coli, which preferably catalyzed the hydrolysis of methionyl peptides, and had a requirement for Mn^{++}. Yaron et al. (256) purified a dipeptidocarboxypeptidase which released dipeptides from the carboxyl end of tetra- or longer peptides. Besides these reports of peptidases from E. coli, some purification of peptidases from Bacillus subtilis (143), and Mycobacterium phlei (175), and naphthylamidases in Sarcina lutea (9) and Neisseria catarrhalis (10) have been reported. Rabier and Desmazeaud (185) purified a dipeptidase and an aminopeptidase from S. thermophilus. The dipeptidase showed an optimum activity around pH 7.5, and was most stable between pH 7.0 and 9.0. The enzyme exhibited metal requirement because of inactivation by EDTA, which was partially reactivated by addition of Co^{++} or Mn^{++}. The enzyme showed a specificity towards dipeptides with a large and hydrophobic amino acid at the amino end of the peptide. The aminopeptidase was most active around pH 6.4, and was stable during storage at pH 5.8 to 9.8. The enzyme was inactivated by EDTA but was totally reactivated by Co^{++} or Mn^{++}. Only amino acids at the amino end of peptides were released from oligopeptides. None of these enzymes showed

any activity toward prolyl peptides. A large number of different peptidases are commonly found in many different bacteria, which suggest that many peptidases also will be present in propionibacteria. These enzymes may have some influence on the ripening of Swiss cheese.

In Swiss cheese proline may be important for flavor, and proline peptidases may be important for proline production. Proteases and peptidases of broad specificity are generally unable to cleave peptide bonds involving proline, necessitating specific enzymes to split such bonds (37). Dehm and Nordwig (37) made a table listing prolyl peptide specific peptidases present in swine kidney (Table 2).

Table 2. Prolyl peptide specific peptidases from swine kidney (Dehm and Nordwig (37))

Enzyme	Bond attacked
Imidodipeptidase (=prolidase)	X↓—pro
X-prolyl-aminopeptidase	X↓—pro---
Iminodipeptidase (=prolinase)	Pro↓—Y
Proline iminopeptidase	Pro↓—Y---
Aminopeptidase cleaving Gly-Pro-β-naphthylamide	X↓—Pro---
Carboxypeptidase P	---Pro↓—Y
Lysosomal carboxypeptidase	---Y↓—Pro

The first report of proline peptidases in bacteria was published by Stone (219) in 1953. He observed that cell extracts of E. coli were able to hydrolyze glycylproline, prolylglycine, and glycylprolylglycine. The first to attempt some purification was Sarid et al. (195), who isolated a proline iminopeptidase from E. coli which is activated by Mn^{++} and acts most rapid on oligopeptides as a specific exopeptidase, cleaving amino terminal proline preferably. An aminopeptidase from Clostridium histolyticum showed the same specificity, but also had a wider specificity (93). Sarid et al. (196) also demonstrated the presence of an iminodipeptidase in cell free extracts of E. coli. Yaron and Mlynar (255) purified aminopeptidase P from E. coli which is related to the X-prolyl-aminopeptidase from swine kidney. This enzyme hydrolyzes only N-terminal proline if it is followed by proline, and the enzyme is activated by Mn^{++}. Ryden (192) purified three proline peptidases from a strain of Arthrobacter. Peptidase A seems to be an iminodipeptidase. Peptidase B also is an iminodipeptidase which shows a wider substrate specificity because it also attacks a few other dipeptides. Peptidase C is an imidodipeptidase. None of these enzymes are activated by metal ions, and they are stable during storage. This shows that most of the enzymes in Table 2 have related enzymes in bacteria. It should be mentioned that iminopeptidases also have been observed in L.

casei, but no activity has been observed in Lactobacillus fermenti, Streptococcus pyogenes, and Streptococcus salivarius (128). Prolinease and prolidase also have been observed in cheese (201, 202).

When one looks at the peptidase activity of E. coli, two facts have been established. Bacteria which have been growing in minimal media possess high levels of peptidase activity, and addition of peptides to the media does not increase the peptidase content significantly (211, 212). These enzymes are also truly intracellular since neither osmotic shock nor spheroplast formation results in significant release of peptidase activity (125, 133, 212).

What is the function of peptidases? Peptidases have a nutritional role, which statement may be supported by the fact that peptides have a superior nutritional value, because they supply several amino acids simultaneously (95, 96). Peptides often accelerate growth in minimal media, and also provide required amino acids in auxotrophic mutants. Kessel and Lubin (92) proved this by showing that a glycine auxotroph lacking diglycine peptidase could not grow on diglycine, even if the transport system is intact.

Peptidases also may have a protective role because they can protect against deleterious effects of peptides. Peptides which accumulate intracellularly inhibit bacterial growth. Meisler and Simmonds (137) observed that certain

leucine peptides prolonged the lag period of E. coli, and that the inhibition is greater for older cells. This inhibitory role may be involved in regulatory control, because such peptides may interfere with the action of physiologically important peptides. Peptides as chemical messengers have not been shown in bacteria, but higher organisms have peptide hormones. As with antibiotics they may contain unusual amino acids to protect them against the peptidases.

Thirdly, peptidases may be involved in protein biosynthesis. Only 45% and 10% of the cellular proteins of E. coli and Bacillus subtilis respectively contain methionine at the amino end. Pine (172) suggests that deformylation takes place prior to peptidase activity, that deformylation takes place during chain elongation as well as after release of the protein from the ribosome, peptidase cleavage occurs after release of the proteins. Matheson et al. (134) and Brown (22) suggest that the ribosome-bound aminopeptidase which shows optimal activity against methionyl peptides is involved. Peptidases also are involved in protein turnover (148). This indicates a number of roles other than in nutrition. The cells also contain the same amount of peptidase if they are grown in a nonpeptide medium which fact also supports the idea of roles other than nutrition. The data suggests that the synthesis of peptidases is constitutive. Simmonds and coworkers (125, 210, 211, 212, 213) proposed that peptidases

exist in a partially latent form in bacteria. This is based on the observation that whole cells display lower peptidase activity than crude extracts from the same cells. The reason for the latency of peptidases of whole cells is unknown, but may be involved in the regulation of the peptidases of which also nothing is known.

Proline biosynthesis

Biosynthesis of proline is the third possible way that proline can be produced in Swiss cheese by propionibacteria. The biosynthetic pathway for proline is shown in Figure 1. The first report of the pathway for proline biosynthesis was published in 1952 by Vogel and Davis (245), and most of the later work has been performed on E. coli. Three enzymes are involved in this pathway. The first enzyme is a glutamic acid kinase which releases P_i from ATP and which is inhibited by proline. The reaction is irreversible (5). The second enzyme is a phosphate-dependent glutamate γ-semialdehyde dehydrogenase which uses $NADP^+$ as a cofactor. The enzyme is not inhibited or repressed by proline, and the reaction is reversible (6). The reversible reaction, however, is more efficient with NAD^+ as a cofactor (53). The third enzyme, pyrroline-5-carboxylate reductase, produces proline by the way of L-γ-pyrroline carboxylate (166). Usually proline is not excreted into the medium indicating regulation of the pathway.

Baich and Pierson (7) showed that feedback-inhibition took place, and that the first enzyme, the kinase, was regulated (6).

By using proline analogues, Baich and Pierson (7) isolated a mutant which excreted proline. Thioproline and 3,4-dehydroproline are especially efficient for producing mutants which excrete proline, which suggests a loss of the allosteric site on the first enzyme of the reaction sequence (52). Mutational techniques could be used for systematic production of a mutant which could be used industrially for proline production, or for increased proline production in Swiss cheese if proline is important for Swiss cheese flavor. However, in both known cases of rapid proline production in the industry, the proline producer was selected by screening hundred of strains of different bacteria (87, 257).

Colorimetric Determination of Proline

The quantitative analysis of proline, a nonessential amino acid, can be done together with other amino acids by automatic amino acid analysis, by gas chromatography, and by extraction of the color spots from paper and thin-layer chromatograms. There has been, however, need for a specific method of analysis for proline in the clinical sciences. Proline is of interest because of hyperprolemia due to a deficiency in proline oxidase in association with hematuria

(46). Hyperprolemia also has been recorded in association with hereditary nephritis. Hyperprolemia causes increased excretion of proline (142). Proline measurement also is important for diagnosis of cystic fibrosis (11), and also has been used for determination of amounts of elastin and collagen in connection with pneunoconiosis (13). In the food sciences, proline measurement may be of specific importance in relationship to the flavor of Swiss cheese.

There are two main colorimetric methods for proline, one based on the reaction with isatin, and the most common based on the ninhydrin reaction. Isatin produces different colors in combination with amino acids in thin-layer and paper chromatography. The characteristic blue color produced by the isatin reaction with proline and hydroxyproline, was first used with column and paper chromatography for quantitative determination of proline after chromatographic separation, spraying with isatin, and elution of the blue spot (115). Boctor (17) developed a spectrophotometric method using isatin as the color reagent. Other amino acids do not interfere, and the method is sensitive. This method has been tested by Langsrud (unpublished results), who found that the reaction product was extremely light sensitive, and even when covered with aluminum foil, absorption decreased rapidly. The color recovered after 5 min in a boiling water bath varied greatly between parallel samples.

This great variation indicates that the more common method using ninhydrin is more accurate. In the early 1930's, Grassmann and von Arnim (62, 63) described a red reaction product of proline and ninhydrin formed in small amounts at pH 7.0. Van Slyke et al. (214) later reported that at about pH 1.0, a red water-insoluble reaction product was formed. No significant amounts of color were produced by other amino acids at pH 1.0.

This information was used by Chinard (27) to develop a colorimetric method for the estimation of proline. An aqueous solution containing proline was heated with a mixture of acetic acid and concentrated phosphoric acid containing ninhydrin to give a red solution. The amino acids ornithine, lysine, hydroxylysine, and cysteine interfere with the method. Most of the recent methods are based on Chinard's method. Schweet (205) observed that Chinard's method gave variable results due to the instability of the colored product. He suggested use of nitrozation to remove the interfering amino acids. Then, modifications were proposed by Troll and Lindsley (236) who claimed that they could remove the basic interfering amino acids by "Decalso", a Permutit cation-exchange resin. They also proposed stabilization of the pigment by extraction with benzene. Subsequently, Finch and Hird (49) reported that amino acids other than those mentioned

by Chinard (27) interfered with the method. These results induced Messer (138) to make a thorough investigation of the effect of 20 amino acids on Chinard's method. Seven gave a color themselves, and ten enhanced the color yield, but did not produce a color. Messer modified the reaction by adding glycine to the solution, and the interference by 10 amino acids and by all peptides tested except glycyl-proline could be eliminated. Sensitivity was increased by 50%. Wren and Wiggall (254) improved Messer's method by including a performic acid oxidation of the protein before hydrolysis, and by removal of basic amino acids on Permutit as suggested by Troll and Lindsley (236).

All of these methods just described share the drawback that the instability of the red pigment in acid solution gives erratic results. Bergman and Loxley (13) overcame this disadvantage by using an aqueous reaction medium with a high electrolyte content, which caused the red pigment to be salted out immediately, thus protecting it against decomposition. This pigment was solubilized with acetic acid. The major interfering amino acids were removed by an improved nitrozatin technique. Goodwin (59) improved this method by extracting the pigment with benzene, improving the sensitivity of the method, and he also modified the method to permit the measurement of about 1 μg/ml of proline. Weak interference was found with arginine in 10 times the amounts of proline.

Some interference was found with glycyl-proline, probably due to hydrolysis during the nitrozation reaction.

Cheese Analysis

Free amino acids in cheese

Extraction of free amino acids from the cheese was mainly performed as described by Kosikowski (109). Six grams of cheese were placed in a Waring blender with about 80 ml of distilled water and the mixture was blended for about 1 minute. The mixture was made up to 100 ml in a graduated cylinder with distilled water and was transferred to a 300-ml Erlenmeyer flask which was heated with constant agitation at 75 C for 10 min, and then cooled to room temperature. The mixture was filtered through Whatman 2V2 filter paper.

To 25 ml of the filtrate was added 125 ml 95% ethanol (221). The solution was agitated and after 30 min filtered through Whatman no. 42 filter paper (11 cm). One hundred ml of filtrate was pipetted into a round-bottom flask, and the alcohol extract was evaporated to dryness in a rotary vacuum evaporator at 40 C. The dried material was solubilized in 12 ml starter buffer, pH 2.87, containing 0.1 µmol norleucine as an internal standard for the amino acid analysis. The samples were frozen and stored for analysis. The amino acid solutions were analyzed on a Technicon Automatic Amino Acid Analyzer, Model TSM (Technicon Instruments Corporation, Tarrytown, N.Y. 10591).

Determination of proline in cheese, milk, media, and bacteria

The method described by Goodwin (59), with a few modifications, was used for determination of the free proline content of cheese, milk, bacteriological media, and for bound proline in nitrogen sources used for bacteriological media. The method was as follows:

Reagents All chemicals used were reagent grade, and all solutions were aqueous except when otherwise specified.

2.5 M Sodium nitrite.

4.8 M Ammonium chloride.

Hydrochloric acid, concentrated.

8.0 M Hydrochloric acid.

5.32 M Orthophosphoric acid.

Phosphate solution. 473.5 g of sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) was dissolved in and diluted to 1000 ml with 5.32 M orthophosphoric acid solution.

10.0 M NaOH.

Ninhydrin solution, 30 gl. Three g/l ninhydrin (1,2,3-indanetrione monohydrate) was dissolved in 100 ml of water, with warming.

The solution was prepared immediately before use.

Protein precipitant. One volume of sodium tungstate stock solution (13.3g/100 ml) was mixed with nine volumes of HCL (0.11 M).

The precipitant was prepared just before use and discarded after 4 h.

Proline stock solution. A solution of 10 mg of L-proline per 100 ml was used.

Working proline standard. Five, 10, 20, and 40 ml portions of proline stock solution was added to four 100 ml volumetric flasks and diluted to volume with distilled water. The standards contained 5, 10, 20, and 40 µg proline/ml.

Deproteination of cheese The preparation of cheese samples was as described for the determination of total free amino acids in cheese up to the start of ethanol precipitation. If the samples contained more proline than 100 µg per ml, 0.2 ml of the sample was pipetted into a 15x125 mm screw-capped test tube. One ml protein precipitant and 4 ml water was added. The sample was mixed and centrifuged at 10,800 x g for 10 min (Sorvall RC2-B refrigerated centrifuge) at room temperature. To measure less than 100 µg proline/ml, the sample size was increased to 1 ml. In this event, 4 ml protein precipitant and 1 ml water were added to the sample before centrifugation. Samples for proline from other sources were treated in the same way.

Nitrozation Five ml of the protein-free samples were pipetted into a 19 x 150 mm test tube. To this sample were added 0.2 ml 8 M HCL and the sample was mixed on a vortex-type mixer. Working standards, 5 ml, containing 5, 10, 20, and 40 µg proline/ml were run simultaneously. 0.5 ml 2.5 M sodium nitrite was added to each tube, mixed and let stand for 10 min. Exactly 0.3 ml 4.8 M ammonium chloride was added, mixed, and allowed to stand at room temperature for 5 min.

Two ml concentrated HCL was added to the tubes, which were capped with aluminum foil, mixed and heated in boiling water for 20 min. Afterwards, the solution was cooled in water, and 2 ml 10 M NaOH were added to the nitrozated solution and mixed.

Color development Two ml nitrozated solution from both samples and standards were added to 19 x 150 mm test tubes. This was followed with 2 ml phosphate solution and 3 ml ninhydrin solution. Each tube was covered with aluminum foil, mixed, and placed in a boiling water bath for heating for 45 min. After cooling, the samples were extracted with 3 ml benzene by mixing in a vortex-type mixer for 2 to 3 min. The phases were allowed to separate. With a Pasteur pipette, the benzene (top) phase was transferred to a test tube. This extraction was repeated with an additional 3 ml of benzene and the extracts were combined. The absorbence was measured at 520 nm in a Bausch & Lomb Spectronic 88 spectrophotometer (Bausch & Lomb, Rochester, New York, N.Y. 14602).

Determination of volatile fatty acids in cheese

Sample preparation Five grams of cheese were blended with 10 ml of distilled water in a test-tube homogenizer. The mixture was acidified with 0.3 ml of concentrated HCl, thoroughly mixed and centrifuged at 10,000 rpm for 10 min using a

Sorvall refrigerated centrifuge (Ivan Sorvall Inc., Newton, Conn.). Two milliliters of the aqueous layer were pipetted into a 19 x 150 mm screw-cap test-tube and 0.2 ml of a 2% valeric acid solution was added to act as an internal standard. The solution was mixed and approximately 1 µl was injected into the gas chromatograph using on-column injection.

Apparatus The apparatus used was an Aerograph series 1520 gas chromatograph (Varian Instrument Division, Palo Alto, Calif.) equipped with a hydrogen flame detector, a glass-lined injection port, and a Honeywell Electronic 15 chart chromatography recorder (Honeywell Industrial Product Group, Philadelphia, Pa.). A 6-ft Telfon column was prepared using 10% Carbowax 400 (Supelco Inc.) and 1% H_3PO_4 terminated on acid washed Chromosorb W (Supelco Inc.) (80/100 mesh). The column packing was dried in a vacuum oven at 100 $\pm$ 5 C for 3 h. Helium was used as the carrier gas.

Instrument conditions The carrier gas flow rate and that of the hydrogen was 40 ml/min. The flow rate of the air was 125 ml/min. The injector temperature was set at 125 C and the detector temperature was set at 150 C. The oven temperature was held at 90 C until the water peak had reached the baseline and then was temperature programmed at 4 C/min to

a maximum of 120 C. Peaks were measured using a Disc integrator (Disc Instruments, Inc., Costa Mesa, Calif.).

Preparation of standard curves Values for standard curves were obtained by injecting known concentrations of a mixture of acetic, propionic, butyric, and valeric acids into the gas chromatograph. The "least squares" method was used to determine the best fitting line for each of the acids.

Calculations of amounts of acids The amount of cheese sample injected into the chromatograph was calculated using the following formulas:

$$\frac{Ws}{Wc} \times \frac{Vs}{Va} \times C_{5a} = C_{5b}$$

where

Ws = total weight of original cheese sample, water, and HCl,

Wc = weight of original sample of cheese,

Vs = 2.2 ml,

Va = 2.0 ml,

C_{5a} = milligrams of valeric acid injected as determined from the standard curve,

C_{5b} = milligrams of valeric acid/g of cheese;

and

$$\frac{C_{5a}}{C_{5b}} = C$$

where

C = total g of cheese injected.

The total grams of cheese injected were then used to determine the amount of acetic, propionic, and butyric acids present in the cheese by the following formula;

$$\frac{FFA}{C} \times 100 = M$$

where

FFA = amount of acetic, propionic, or butyric acid injected as determined from the standard curves,

C = total g of cheese injected, and

M = milligrams of acid present in 100 g of cheese.

Proline Production by Propionibacteria

Proline production in milk by *P. shermanii*, P-59

Reconstituted dry milk was used in this experiment. To enhance growth, small amounts of either Trypticase, yeast extract, glucose, or sodium lactate were added alone or in different combinations.

These media were dispensed in 10-ml amounts in 18 x 125 mm screw-capped test tubes and were autoclaved at 121 C for 15 min. The tubes were incoculated with 1 drop of a 48-h-old culture of *P. shermanii*, P-59, and incubated at 32 C. Free proline contents and total bacterial cell numbers were determined after 0, 1, 2, 3, 4, 6, 15, and 35 days. Proline

was determined on 1-ml samples as described earlier. Total numbers of propionibacteria were determined by the pouch method of Hettinga et al. (79).

Effect of yeast extract (Difco) on proline production from Trypticase (BBL) by P. shermanii, P-59

Three media combinations were used; 1. Sodium lactate broth (SLB) without Trypticase (BBL); 2. SLB; and 3. SLB in which the yeast extract had been exchanged with a vitamin and salt solution as described for the defined medium. The pH of each medium was adjusted to pH 6.8. The adjusted media were dispensed in 10-ml amounts in 18 x 125 mm screw-capped test tubes and autoclaved at 121 C for 15 min. The media were then inoculated with 1 drop of a 48-h-old culture of P. shermanii, P-59, grown in SLB. The inoculated media were incubated at 32 C. Bacterial cell numbers were determined after 0, 2, 3, 4, 7, and 15 days, and proline after 3, 7, 15, and 35 days. Media pHs were measured throughout each experiment.

Total amount of proline in different nitrogen sources

The total amount of proline was determined in each of: 1. Yeast extract (Difco); 2. Typticase (BBL); 3. Tryptone (Difco); 4. Proteose peptone (Difco); 5. Proteose peptone No. 3 (Difco); 6. Phytone-peptone (BBL); 7. Protone (Difco); 8. Peptone (Difco); and 9. Casitone (Difco). Approximately

50 mg of each nitrogen source were solubilized in 6 ml 6 N HCl. Five ml were transferred to a freeze-drying tube which was sealed. Hydrolysis was induced in an autoclave at 106 C for 24 h. After hydrolysis, the solution was transferred to a 25-ml beaker, and the freeze-drying tube was washed with 1 ml 6N HCl. The hydrolyzate was dried under vacuum in a desiccator over NaOH at 32 C to dryness. The hydrolyzate was solubilized in warm water, filtered, again evaporated to dryness (209), and finally taken up in 10 ml warm water. These samples were analyzed for proline.

Effect of different nitrogen sources on proline production by P. shermanii, P-59

Commercial enzymatic hydrolysates of casein, meat, and plant proteins with different amounts of peptides, and also, variations in peptide size were used as nitrogen sources (21, 40). The different media used are shown in Table 3. All media were adjusted to pH 6.8 and dispensed in 10-ml amounts in 18 x 125 mm screw-capped test tubes, and autoclaved at 121 C for 15 min. One drop of a 48-h-old culture of P. shermanii was added to each tube and the inoculated media were incubated at 32 C. Bacterial cell numbers were determined after 0, 1, 2, 3, 4, 8, and 15 days. Proline contents were determined after 0, 2, 3, 4, 8, 15, 35, and 75 days on 0.2-ml samples.

Table 3. Different media combinations[a]

Source of hydrolysate	Brand name	Amounts added
Casein	Casitone, Difco	10 g/l
	Trypticase, BBL	10 g/l
	Tryptone, Difco	10 g/l
Meat	Peptone, Difco	10 g/l
	Proteose-peptone, Difco	10 g/l
	Proteose-peptone No. 3, Difco	10 g/l
Plant	Phytone-peptone, BBL	10 g/l
Mixed	Protone, Difco	10 g/l

[a]Control medium: Yeast extract, 10 g/l, sodium lactate (70%) 16.2 ml/l, dipotassium hydrogen phosphate, 0.250 g/l, water, 1 l.

Effect of addition of long-chain peptides and casein to SLB on proline production by P. shermanii, P-59

The same control medium was used as reported in the previous experiment. In addition to Trypticase (BBL), an enzymatic hydrolysate, Protone (Difco), containing long-chain peptides, or casein were added to the basal medium. The media combinations are shown in Table 4.

The treatment of the media, inoculation level of P. shermanii, P-59, and incubation were as for the previous experiment. Sampling times for proline and total cell numbers were as described earlier.

Table 4. Different media combinations[a]

Nitrogen source	Amounts added
Trypticase, BBL	10 g/l
Trypticase, BBL Protone, Difco	10 g/l 1 g/l
Typticase, BBL Protone, Difco	10 g/l 5 g/l
Trypticase, BBL Casein, vitamin-free, NBC	10 g/l 1 g/l
Trypticase, BBL Casein, vitamin-free, NBC	10 g/l 5 g/l

[a]Control medium: yeast extract, 10 g/l, sodium lactate (70%) 16.2 ml/l, dipotassium hydrogen phosphate, 0.250 g/l, water 1l.

Influence of different concentrations of Trypticase (BBL) in SLB on proline production by P. shermanii, P-59

The control medium was SLB without Trypticase (BBL). Seven different media combinations were prepared by adding the following amounts of Trypticase: 1. 1 g/l; 2. 5 g/l; 3. 10 g/l; 4. 20 g/l; 5. 30 g/l; 6. 40 g/l; and 7. 50 g/l, and the pH in each instance was adjusted to pH 6.8. The media were dispensed in 10 ml amounts in 18 x 125 mm screw-capped test tubes, and were autoclaved at 121 C for 15 min. Each tube of medium was inoculated with 1 drop of a 48-h culture of P. shermanii, P-59, grown in SLB. The inoculated media were incubated at 32 C and were sampled for

total cell numbers after 3 days. Proline contents were determined after 15, 35, and 75 days. pH was measured throughout the experiment.

Effect of Peptone (Difco) on proline production from Trypticase (BBL) by P. shermanii, P-59

The control medium used was SLB without addition of Trypticase (BBL). Media containing different amounts of Trypticase (BBL) and Peptone (Difco) were prepared as shown in Table 5.

Table 5. Media composition

Media no.	Trypticase g/l	Peptone g/l
1	1	0
2	5	0
3	10	0
4	30	0
5	1	10
6	5	10
7	10	1
8	10	5
9	10	10
10	10	30
11	30	10
12	0	1
13	0	5
14	0	10
15	0	30

The media were dispensed in 10-ml amounts in 18x125 mm screw-capped test tubes, autoclaved, and inoculated with 1 drop of a 48-h-old culture of P. shermanii, P-59, grown in

SLB. Total cell numbers were determined after 3 days, and proline after 7, 15, and 35 days. pH was measured throughout the experiment.

Growth studies of five strains of propionibacteria in medium

The medium composition is shown in Table 6. The medium was prepared by mixing 20 ml of salt solution I, 20 ml of salt solution II, 2 ml each of amino acid solution I to V, and 1 ml of a 10% carbohydrate solution. One ml of each vitamin solution was added and the solution was made up to 100 ml and autoclaved in the usual manner.

The purpose of this experiment was to decide which vitamins were necessary for optimum growth of five strains of propionibacteria used for cheesemaking. The following strains were used: P. shermanii, P-59, P. freudenreichii, P-19, P. shermanii, P-24, Propionibacterium zeae, P-35, and Propionibacterium pentosaceum, P-9. The bacteria were grown in 10 ml SLB, centrifuged at 6000 x g in a Sorvall RC2B refrigerated centrifuge, and washed twice with a 0.9% sterile saline solution. One drop of washed cells was inoculated in 4 ml medium in a 10 x 120 mm test tube. The following media combinations were used: 1. Control (defined medium without any vitamins), 2. Control plus thiamin, biotin, Ca-panthotenate, and riboflavin, 3. Number 2 plus nicotinamide, 4. Number 3 plus meso-inositol, 5. Number 4 plus p-aminobenzoic acid, 6.

Table 6. Defined medium for the genus Propionibacterium

Salt solution I	Conc. in medium (g/l)	Conc. in stock solution (g/1.5 l)
CH_3COONH_4	4.00	30.0
$Na_2HPO_4 \cdot 2H_2O$	1.20	9.0
KH_2PO_4	1.20	9.0
Salt solution II	**Conc. in medium (g/l)**	**Conc. in stock solution (g/1.5 l)**
$MgSO_4 \cdot 7H_2O$	0.60	4.500
$FeSO_4 \cdot 7H_2O$	0.02	0.150
$MnCl_2 \cdot 4H_2O$	0.01	0.075
Carbohydrate	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
Glucose	4.00	40.00
Amino acid solution I	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
Glycine	0.05	0.25
L-alanine	0.10	0.50
L-phenylalanine	0.10	0.50
L-valine	0.10	0.50
L-leucine	0.10	0.50
L-arginine HCl	0.10	0.50
L-aspartic acid	0.09	0.45
L-glutamic acid	0.20	1.00
L-tryptophan	0.10	0.50
Amino acid solution II	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
L-isoleucine	0.10	0.50
L-lysine HCl	0.10	0.50
L-histidine HCl	0.10	0.50
L-methionine	0.10	0.50
L-serine	0.10	0.50
L-threonine	0.10	0.50

Table 6 (Continued)

Amino acid solution III	Conc. in medium (g/l)	Conc. in stock solution (g/100 ml)
L-tyrosine	0.10	0.50
Amino acid solution IV	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
L-cystine	0.05	0.25
Amino acid solution V	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
L-proline	0.10	0.50
Vitamin solutions I	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
Thiamine	0.02	0.20
Biotin	0.02	0.20
Ca-pantothenate	0.02	0.20
Riboflavin	0.02	0.20
Vitamin solutions II	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
Nicotinic acid	0.02	0.20
Vitamin solutions III	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
Meso-inositol	0.02	0.20
Vitamin solutions IV	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
p-Aminobenzoic acid	0.02	0.20
Vitamin solutions V	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
Pyridoxal phosphate	0.02	0.20

Table 6 (Continued)

Vitamin solutions VI	Conc. in medium (g/l)	Conc. in stock solution (g/100 ml)
Folic acid	0.02	0.20
Vitamin solutions VII	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
Ascorbic acid	0.02	0.20
Vitamin solutions VIII	**Conc. in medium (g/l)**	**Conc. in stock solution (g/100 ml)**
Choline chloride	0.02	0.20

Amino acid solution III and IV were solubilized by addition of 0.83 ml concentrated HCl. The pH of each solution was adjusted to pH 6.8 before sterilizing. All solutions except for the vitamin solutions were autoclaved at 121 C for 20 min. The vitamin solutions were filter-sterilized. Pyridoxal phosphate was protected from light. To make 100 ml of medium, 20 ml of salt solution I, 20 ml of salt solution II, 1 ml of carbohydrate stock, 2 ml of each of the amino acid solutions, and 1 ml of each of the vitamins were mixed together, and the volume was made up to 100 ml with addition of sterile water. The sterile medium was then aseptically distributed into sterile tubes (3-5-10 ml, etc.), and was thus ready for use.

Number 5 plus pyridoxal phosphate, 7. Number 6 plus folic acid, 8. Number 7 plus ascorbic acid, and, 9. Number 8 plus choline chloride.

Growth was measured after 0, 24, 48, and 72 h at 540 nm in a Bausch & Lomb Spectronic 88 spectrophotometer.

Proline production in defined medium by P. shermanii, P-59

The defined medium was prepared as described earlier with the exception that glucose was used as the energy source, and only vitamin solutions no. 1, 4, and 5 were added. One medium contained proline, while the other did not. Propionibacterium shermanii, P-59, was transferred three times in defined medium before the experiment started. One drop of a 72-h-old culture was added to 10 ml sterile defined medium in 18 x 125 mm screw-capped test tubes. Samples and analysis for total free proline and optimum cell numbers were determined as described earlier. One-ml samples were used for the proline determinations.

Effect of different concentrations of Casamino acids (Difco) on proline production by P. shermanii, P-59

A basal medium containing the salts of the defined medium described earlier and 10 g/l sodium lactate plus 0.02 g/l of the following vitamins: thiamine, biotin, Ca-panthothenate, riboflavin, pyridoxal phosphate, and folic acid was used. Seven different media combinations were made by adding the following amounts of Casamino acids (Difco): 1 g/l, 5 g/l, 10 g/l, 20 g/l, 30 g/l, 40 g/l, and 50 g/l, and the pH of each medium was adjusted to pH 6.8. The different media were dispensed in 10-ml amounts into 18 x 125 mm screw-capped test tubes which were autoclaved at 121 C for 15 min. Each

tube of medium was inoculated with 1 drop of a 72-h-old culture of P. shermanii, P-59, transferred three times in a defined medium. The inoculated tubes of media were incubated at 32 C and were sampled for total cell numbers after 4 days, and for proline after 15, 35, and 75 days. pH was measured throughout the experiment.

Proline production in a medium with low concentrations of Trypticase (Difco) by P. shermanii, P-59

The basal medium, which was divided into seven parts, contained a vitamin and salt mixture and sodium lactate as described earlier. The following amounts of Trypticase (BBL) were added, respectively, to each part: 1. 1g/l; 2. 2.5 g/l; 3. 5 g/l; 4. 7.5 g/l; 5. 10 g/l; 6. 12.5 g/l; and, 7. 15 g/l. The media were dispensed in 10-ml amounts and autoclaved at 121 C for 15 min. The tubes of media were inoculated with 1 drop of a 48-h-old culture of P. shermanii, P-59, and incubated at 32 C. Cell numbers were determined after 3 days, and proline after 3, 7, 15, 35, and 75 days. The amounts of free proline produced from Trypticase were compared with the total amounts of peptide-bound proline in Trypticase. pH was measured throughout the experiment.

Effect of glutamic acid on proline production by P. shermanii, P-59

The basal medium contained defined amounts of salts, vitamins, and sodium lactate as described earlier, to which was added 5 g/l of Casamino acids (Difco). The following amounts of glutamic acid were added to make five media: 1. 0.5 g/l; 2. 1 g/l; 3. 2 g/l; 4. 4 g/l; and 5. 8 g/l. The media were dispensed in 10-ml amounts in 18 x 125 mm screw-capped test tubes and autoclaved at 121 C for 15 min. The tubes of media were inoculated with 1 drop of a 72-h-old culture of P. shermanii, P-59, grown in defined medium, and incubated at 32 C. Bacterial cell numbers were determined after 0, 3, 5, 7, and 15 days, and proline after 3, 7, 15, and 35 days. pH was measured throughout the experiment.

Effect of arginine on proline production by P. shermanii, P-59

The basal medium was described earlier for the experiment on the effect of glutamic acid on proline production. Instead of glutamic acid, the following amounts of arginine were added to the medium: 1. 0.5 g/l; 2. 1 g/l; 3. 2 g/l; and, 4. 4 g/l. Treatment, inoculation, and incubation were as before. Bacterial cell numbers were determined after 0, 3, 5, 7, and 15 days, and proline after 3, 7, and 15 days.

Proline production from casein by P. shermanii, P-59

The control medium contained defined amounts of salts, vitamins, and sodium lactate to which was added 5 g/l of Casamino acids (Difco). The following amounts of casein were added to make three media: 1. 1 g/l; 2. 5 g/l; and 3. 10 g/l. The media were dispensed in 10-ml amounts and autoclaved at 121 C for 15 min. The tubes of media were inoculated with 1 drop of a 72-h-old culture of P. shermanii, P-59, grown in defined medium, and incubated at 32 C. Bacterial cell numbers were determined after 0, 3, 5, and 7 days, and proline after 3, 7, 15, and 35 days. pH was measured throughout the experiment.

Free proline in the amino acid pool of P. shermanii, P-59

Determination of proline in the amino acid pool of P. shermanii, P-59, was performed on cells grown in 50 ml SLB. The medium was autoclaved at 121 C for 15 min before inoculation with 0.5 ml of a 48-h-old culture, followed by incubation at 32 C. Analyses were done after 1, 2, 3, 4, and 5 days for free proline in the medium, for free proline in the amino acid pool, for protein-bound proline in the cells, and for protein-bound proline released into the medium. Total cell numbers also were determined. The cells were removed by centrifugation at 35,000 x g for 1 min at 0 C in a Sorvall RC2B refrigerated centrifuge.

The supernatant was removed and was used for proline analysis and for determination of released protein-bound proline. The protein was precipitated from 10 ml of supernatant by addition of 10 ml 5% trichloroacetic acid, isolated by centrifugation at 10,000 x g for 10 min, solubilized in 6 ml of 6 N HCl, and 5 ml of the final material was transferred to a hydrolysis tube which was sealed and hydrolyzed as described earlier.

After removal of the supernatant, the cells were washed twice with 0.1 M phosphate buffer, pH 7.5 (207). Before the last wash, the cell solution was divided into two equal parts. After the last wash the cells were weighed. One part was extracted with 10 ml 10% (w/v) cold trichloroacetic acid at 0 C for 30 min with frequent stirring. The cell debris was removed by centrifugation at 10,000 x g for 5 min at 0 C. The clear supernatant (10 ml) was then extracted with 1 ml of cold ether for 5 min in a separation funnel after which the ether was removed. The extraction was repeated until the pH of the supernatant was 5.0. Finally, the solution was neutralized with 0.01 N KOH (227). One ml of the neutral solution was used for proline analysis. To the second part 6 ml of 6 N HCl were added and 5 ml of the combined solution where transferred to a hydrolysis tube, hydrolyzed, and then analyzed as described earlier.

Proline Production by Different Strains of Propionibacteria, and Effect of different Conditions Related to Cheese Ripening on Proline Production

Proline production by different strains of propionibacteria

The propionibacterial strains described in Table 7 were used. Frozen stock cultures were obtained from the culture collection of the Department of Food Technology. The cultures were rapidly thawed in a waterbath at 37 C, and then directly streaked on Sodium lactate agar plates. These plates were incubated in a candle oats jar for 5 days at 32 C (238). Single colonies were picked and transferred into tubes containing sterile SLB (10 ml) which were incubated at 32 C for 48 h. The different cultures were gram-stained, checked for catalase activity, and for carbohydrate fermentation patterns (20). The cultures were activated before the experiment by transferring three times every 48th h. One drop of a 48-h-old culture was added to 10-ml SLB, and the inoculated tubes were incubated at 32 C for 35 days. Cell numbers were determined after 3 days, and proline after 15 and 35 days. pH was measured throughout the experiment.

Effect of growth temperature on proline production by *P. shermanii*, P-59

Sodium lactate broth, pH 6.8, was dispensed in 10-ml amounts, autoclaved, and inoculated with 1 drop of a 48-h-old culture of *P. shermanii*, P-59. The inoculated tubes

Table 7. *Propionibacterium* strains used in the investigation

Species[a]	Source[b]	ISU no.	Strain no.	Original designation from source
P. freudenreichii	C	P-19	KP 1	*P. shermanii*
P. freudenreichii	O	P-89	5571	*P. freudenreichii*
P. freudenreichii	F	P-104	ATCC 9614	*P. freudenreichii*
P. intermedium	K	P-74	PZ 99	*P. zeae*
P. intermedium	F	P-106	ATCC 4964	*P. zeae*
P. jensenii	J	P-52	E.5.1	*P. petersonii*
P. pentosaceum	B	P-9	129	*P. arabinosum*
P. pentosaceum	H	P-42	10	*P. arabinosum*
P. pentosaceum	J	P-50	E.7.1	*P. arabinosum*
P. pentosaceum	F	P-58	ATCC 4875	*P. pentosaceum*
P. pentosaceum	K	P-79	PT 52	*P. thoenii*
P. pentosaceum	O	P-90	5578	*P. pentosaceum*
P. raffinosaceum	A	P-25	J 17	*P. jensenii*
P. raffinosaceum	F	P-107	ATCC 14073	*P. technicum*
P. rubrum	A	P-10	R 9611	*P. rubrum*
P. rubrum	A	P-17	R 19	*P. rubrum*
P. rubrum	F	P-105	ATCC 4871	*P. rubrum*
P. rubrum	F	P-108	ATCC 14072	*P. intermedium*
P. shermanii	A	P-1	F 24	*P. freudenreichii*
P. shermanii	A	P-11	P 31 C	*P. pentosaceum*
P. shermanii	C	P-24	KP 2	*P. shermanii*

[a]Species classified according to R. V. Ogden. 1974. Classification and DNA base ratios of propionibacteria. Unpublished Ph.D. Thesis. Iowa State University. Ames, Iowa. 154 pp.

[b]Sources: A. Cornell University, Ithaca, N.Y.; B. Iowa State Univeristy, Ames; C. Kraft Foods Co., Stockton, Ill.; E. Dr. K. W. Sahli, Station Federale D'Industrie Laitiere Liebefeld-Bern, Switzerland; F. American Type Culture Collection, Rockville, Md.; H. Dr. W. Kundrat, University of Munich, Munich, Germany; J. Dr. C. B. van Niel, Hopkins Marine Station, Pacific Grove, Calif.; K. Communicable Disease Laboratory, Atlanta, Ga.; L. Isolated from Gruyére cheese imported from France; O. Origin unknown.

Table 7 (Continued)

Species[a]	Source[b]	ISU no.	Strain no.	Original designation from source
P. shermanii	B	P-59	ISU	P. shermanii
P. shermanii	B	P-83	SAUER	P. shermanii
P. shermanii	F	P-98	ATCC 9614	P. shermanii
P. thoenii	A	P-4	TH 25	P. thoenii
P. thoenii	A	P-15	TH 20	P. thoenii
P. thoenii	A	P-20	Th 21	P. thoenii
P. thoenii	J	P-53	E.5.2.	P. petersonii
P. zeae	E	P-35	1505	P. petersonii
P. zeae	J	P-46	E.1.2.	P. jensenii
P. zeae	J	P-54	E.1.1.	P. jensenii
P. zeae	L	P-86	11	P. zeae

were incubated at 15, 21, 27, 32, and 37 C for 75 days. Cell numbers were determined after 2 and 3 days at 32 and 37 C, after 3 and 4 days at 27 C, after 3, 4, 5, and 6 days at 21 C, and after 7, 14, and 21 days at 15 C. Proline contents were determined after 7, 15, 35, and 75 days as earlier described. pH changes were recorded.

Proline production in SLB at 21 C by six strains of propionibacteria

The proline-producing capacity of strains of propionibacteria (1. P. freudenreichii, P-15; 2. P. shermanii, P-24; 3. P. shermanii, P-59; 4. P. shermanii, P-1; 5. P. zeae,

P-35; and 6. P. pentosaceum, P-9), all commonly used in Swiss cheese manufacture, were measured after growth at 21 C. Ten ml-amounts of autoclaved SLB in 18 x 125 mm screw-capped test tubes were inoculated with 1 drop of a 48-h-old culture grown at 32 C. The inoculated tubes of medium were incubated at 21 C. Total cell numbers were determined after 6 days, and proline was determined after 15, 35, 75, and 120 days. pH was measured during the experiment.

Proline production in SLB at 3 C by six strains of propionibacteria

The proline-producing capacity of the same six strains of propionibacteria as in the last experiment was followed at 3 C. Ten-ml amounts of autoclaved SLB were inoculated with 1 drop of a 48-h-old culture grown in SLB at 32 C. The inoculated tubes of media were incubated at 32 C for 6 days, rapidly cooled, and transferred to a 3 C incubator where they were stored. After 3 days at 32 C, the inoculated tubes were sampled for total cell numbers. Proline contents were determined after 6, 15, 35, 75, and 120 days. pH was measured during the experiment.

Effect of initial medium pH on proline production by P. shermanii, P-59

Sodium lactate broth was prepared and divided into four parts. These four parts were adjusted to pH 6.8, 6.0, 5.5, and 5.2 with 0.1 N HCl. These media were dispensed in 10-ml amounts in 18 x 125 mm screw-capped test tubes and autoclaved at 121 C for 15 min. The media were inoculated with 1 drop of a 48-h-old culture of P. shermanii, P-59, grown in SLB. The inoculated tubes of media were incubated at 32 C. Cell numbers were determined after 0, 2, 3, 4, 7, and 15 days, and proline after 3, 7, 15, and 35 days. pH was measured throughout the experiment.

Effect of NaCl on proline production by P. shermanii, P-59

Sodium lactate broth was prepared and divided into five parts. Sodium chloride was added to each part in the following amounts: 1. 0 g/l; 2. 1 g/l; 3. 5 g/l; 4. 10 g/l; and, 5. 15 g/l. The media were dispensed in 10-ml amounts in 18 x 125 mm screw-capped test tubes and autoclaved at 121 C for 15 min. The media were inoculated with 1 drop of a 48-h-old culture of P. shermanii, P-59, and incubated at 32 C. Cell numbers were determined after 0, 2, 3, 4, 7, and 15 days, and proline after 3, 7, 15, and 35 days. pH was measured throughout the experiment.

Effect of copper on growth and proline production by P. shermanii, P-59

Eight ppm and 16 ppm Cu^{++} were added to reconstituted nonfat dry milk fortified with 0.1 g/l sodium lactate, 0.1 g/l Trypticase, and 0.1 g/l yeast extract, and to SLB. Controls without copper were used for all experiments. The different media were dispensed in 10-ml amounts and autoclaved, and 1 drop of a 48-h-old culture of P. shermanii, P-59, was added to each tube of medium. Media were incubated at 32 C. Cell numbers and proline content were determined as previously described.

Autolysis and Proline Production by Propionibacterium

Preparation of cell-free extracts of propionibacteria

A 1% inoculum of actively growing culture of P. shermanii, P-59, was added to 2 l SLB which was incubated at 32 C. The cells were harvested after 48 h in a Sorvall RC2B refrigerated centrifuge at 6000 x g for 15 min in a large rotor at 4 C. The cells were washed twice in 0.05 M phosphate buffer, pH 7.0, and were then suspended in the buffer to obtain a thick slurry. The cell slurry was subjected to 18,000 psi in a French Press (Aminco, Inc., Silver Springs, Maryland) to obtain initial disruption of cells. Two passages through the French Press accomplished

good disruption of the cells. Separation of the cell-free extract from cell debris was accomplished by centrifugation at 37,000 x g at 4 C. The cell-free extract was decanted from the cell debris and separated into 2-ml portions and placed in screw-capped test tubes. The cell-free extract was stored at -20 C until used for analysis.

Proline imino- and imidopeptidase in P. shermanii, P-59

Cell-free extracts from P. shermanii, P-59, were used for this experiment.

Assay Substrates for this experiment were L-Pro-Gly for proline iminopeptidase, and Gly-L-Pro for proline imidopeptidase. The procedure consisted of adding together 1 ml 0.05 M potassium phosphate buffer, pH 7.0, 0.1 ml co-factor or water, and 0.4 ml 37.5 mM substrate in water. To this was added 0.5 ml or less of the cell-free extract. The total volume was adjusted to 2 ml with water. Enzyme activity was assayed at 32 C, and was sampled (0.1 to 0.2 ml) after 15 and 30 min, and after 1, 2, 3, and 6 h.

Messer's method (138) for proline was used. The sample was transferred to a test tube, to which was added 0.9 or 0.8 ml water. One ml of a 3.6 mM aqueous glycine solution, 1 ml glacial acetic acid, and 1 ml of a 2% ninhydrin solution

freshly prepared in a mixture of four volumes of 6 M ortho-phosphoric and 6 volumes of glacial acetic acid, were added and mixed. Each mixture was heated in a boiling water bath for 30 min, and was rapidly cooled. One ml glacial acetic acid was added and the color was read at 515 nm in a Bausch & Lomb Spectronic 80 spectrophotometer.

Influence of cofactors Instead of 0.1 ml water, 0.1 ml of 0.05 M cobalt chloride or 0.05 M manganese chloride, or 0.05 M NaCl was added. Enzyme activity was compared with activity obtained without cofactors. The assay was performed at 32 C.

Influence of pH A series of phosphate buffers (0.05 M) with pHs varying from pH 5.6 to pH 7.6, and an acetate buffer (0.05 M) pH 5.2 and 5.6 were used. The assay was run at 32 C.

Influence of other proline peptides In addition to L-Pro-Gly and Gly-L-Pro the following substrates were tested: L-Pro-L-Phe, L-Pro-L-Ala, L-Pro-L-Leu, L-Phe-L-Pro, and L-Ala-L-Pro.

Dipeptidase activity in P. shermanii, P-59

The same cell supernatant as was used for the proline iminopeptidase experiment was used. Assay conditions were the same, except that the following substrates were used:

L-Leu-Gly, L-Leu-L-Tyr, Gly-L-Tyr, Gly-L-Leu, Gly-L-Try, Gly-Gly, Gly-Gly-Gly, and L-Leu-Gly-Gly. For measurement of released amino acids, the trinitrobenzene sulphonate (TNBS) method of Binkley et al. (14), as described by Payne (162), was used. A 1-ml sample was added to 4.5-ml of TNBS-Cu^{++} reagent (0.9 ml TNBS (4 mg/ml), 3.42 ml 5% sodium borate decahydrate, and 0.18 ml 0.6% cupric sulfate), and the mixture was incubated at 37 C for 20 min. Exactly 0.1 ml 11.6 M HCl was immediately added to the solution, which was mixed and the extinction was read in a Bausch and Lomb Spectronic 80 at 420 nm. The influence of Co^{++} and Mn^{++}, as described for proline peptidases, was also included.

Autolysis of P. shermanii, P-59

P. shermanii, P-59, was grown in 10-ml amounts of autoclaved SLB at 32 C. Cells were harvested in a refrigerated Sorvall RC2B centrifuge at 6000 x g for 15 min at 2 to 4 C. The cells were washed once in 0.9 % NaCl, and then twice in 0.1 M Tris-HCl buffer containing 0.01 m $MgCl_2$ (pH 7.5). The cells were resuspended in the buffer used for the specific autolysis experiment. Initial optical density was adjusted to 1.0 in a Bausch & Lomb Spectronic 80 spectrophotometer at 420 nm using 4 ml of suspension in 10-mm cuvettes calibrated for direct reading. The cuvettes

were incubated in a water bath at 32 C, and the decrease in optical density was measured (149).

Influence of ionic strength of buffers on autolysis of P. shermanii, P-59 Autolysis was measured in potassium phosphate buffer at pH 7.2 at the following ionic strengths: I = 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.5, 0.75, 1.0, and 1.25. Deionized water was used as control. Autolysis of 24, 48, and 72-h-old cells was measured.

Influence of pH on autolysis of P. shermanii, P-59 Autolysis was measured in phosphate buffers ranging from pH 5.6 to 7.6, and acetate buffers from pH 5.2 to 5.6 at a constant ionic strength of 0.3 at 32 C. Optical density at 440 nm was measured after 0, 3, 6, 9, and 12 h.

Autolysis of P. shermanii, P-59, in a salt solution

A salt solution simulating a milk ultrafiltrate as described by Jenness and Koops (85) was used. Two stock solutions were prepared:

Solution 1:	KH_2PO_4	15.80 g
	K_3 citrate·H_2O	12.00 g
	Na_3 citrate·$5H_2O$	21.20 g
	K_2SO_4	1.80 g
	KCl	6.00 g

Solution 2:	$CaCl_2 \cdot 2\ H_2O$	13.19 g
	$MgCl_2 \cdot 6\ H_2O$	6.50 g

Each of the stock solutions were dissolved in 200 ml distilled water. The salt solution was prepared by adding 20 ml of stock 1 and 20 ml of stock 2 to 940 ml distilled water, and 0.30 g K_2CO_3 was added. Two of these solutions were prepared and pHs were adjusted, respectively, to pH 5.2 and 5.6, and the volumes were made up to 1 l. The ionic strength of these solutions was calculated as described by Munro (147) and was found to be about 0.3. Cells of P. shermanii, P-59, were treated as described earlier and autolysis was followed at 32 C.

Autolysis of seven strains of Propionibacterium

The following strains of propionibacteria grown in SLB at 32 C for 72 h were used: 1. P. freudenreichii, P-19; 2. P. shermanii, P-24; 3. P. shermanii, P-59; 4. P. shermanii, P-1; 5. P. zeae, P-35; 6. P. pentosaceum, P-9; and, 7. P. intermedium, P-74. Autolysis was measured at 32 C in phosphate buffer, pH 7.2, at an ionic strength of 0.3.

Autolysis of P. shermanii, P-59, during growth in different media

Four different media were used in this experiment. One medium was SLB. The other media were prepared from a basal

medium containing the salts of the defined medium as described earlier, 10 g/l sodium lactate and 0.02 g/l of the following vitamins: thiamin, biotin, Ca-pantothenate, riboflavin, pyridoxal phosphate, and folic acid. One medium contained 10 g/l Trypticase, another 5 g/l Casamino acids, and the last contained 20 g/l Casamino acids. The four media were dispensed in 10-ml amounts into 18 x 125 mm screw-capped test tubes which were autoclaved at 121 C for 15 min. Each tube of medium was inoculated with 1 drop of a 48-h-old culture of P. shermanii, P-59, grown in SLB. Total cell number and optical densities at 660 nm in a Bausch and Lomb Spectronic 80 spectrophotometer were determined after 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, and 14 days.

Differences in autolysis between different strains of Propionibacterium during growth in SLB

The autolysis of the following strains of Propionibacterium: P. freudenreichii, P-19, P. shermanii, P-24, P. shermanii, P-59, P. shermanii, P-1, P. zeae, P-35, P. pentosaceum, P-9, and P. intermedium, P-74, were measured. Ten-ml amounts of autoclaved SLB in 18 x 125 mm screw-capped test tubes were inoculated with 1 drop of a 48-h-old culture grown in SLB, and incubated at 32 C. Total cell numbers and optical densities were measured after 0, 1, 2, 4, 5, 6, 8, 10, and 14 days. Optical density was

read at 660 nm in a Bausch and Lomb Spectronic 80 spectrophotometer.

Proline production in relation to cell autolysis

P. shermanii, P-59, and P. pentosaceum, P-9, were grown in 50-ml amounts of SLB at 32 C. Analyses were performed after 1, 2, 3, 4, 5, 7, and 14 days. The following analyses were made:

1. Number of viable cells.
2. Optical density.
3. Proline content.
4. Proline iminopeptidase.
5. RNA content of medium.
6. DNA content of medium.

1. The numbers of viable cells/ml were determined by the pouch method (79).

2. Optical densities were determined at 660 nm as described earlier. Afterwards the cells were removed by centrifugation at 30,000 x g for 1 min at 0 C. The supernatant was used for the remainder of the analyses.

3. 0.2 ml of the supernatant was used for proline determination as described earlier.

4. Five ml of each supernatant were dialyzed for 24 h at 0 C against a 0.05 M phosphate buffer, pH 7.0, to remove interfering compounds. The assay was performed as earlier

described, except that 0.5 ml of each supernatant was used.

5. RNA released into the medium. For this analysis, the orcinol method, originally suggested by Militzer (139) and modified by Herbert et al. (73) was used. By using a strict 20 min heating time, ribose can be used as a standard, but if the heating requirements are not observed, the standard used has to be RNA. A ribose standard was used in this experiment. Reagents:

(I) 0.90 g $FeCl_3 \cdot 6H_2O$ in 1 l of conc. HCl (sp. g. = 1.186)

(II) 1 g orcinol in 100 ml of distilled water

(III) Orcinol reagent. One volume reagent (II) was added to four volumes reagent (I) immediately before use

The orcinol reaction was conventionally carried out in glass-stoppered test tubes graduated to 15 ml. Into these was measured 1 ml of sample (3-120 µg ribose), followed by 3 ml of freshly prepared orcinol reagent (III). Reagent blank and standard ribose solutions were also prepared. Heating was in boiling water bath for 20 min, cooling in tap water, and the amounts were made up to 15 ml with analytical grade n-butanol. Color was read in a spectrophotometer at 672 nm using 1-cm cuvettes for the range 20 to 120 µg, and 4-cm cuvettes for the range below 20 µg.

6. DNA released into the medium. Burton's method (24) was used. Reagents:

(I) DNA standards. A pure DNA preparation (e.g., calf thymus) was dissolved in 5 mM NaOH to give 400 µg/ml; this should be stable at 4 C for 6 months. Working standards were prepared every 3 weeks by mixing a measured volume of the stock standard with an equal amount of 1 N $HClO_4$, and heating for 15 min at 70 C.

(II) Diphenylamine reagent. This was prepared by dissolving 1.5 g steam-distilled diphenylamine in 100 ml of redistilled glacial acetic acid and adding 1.5 ml conc. H_2SO_4. It was stored in the dark. On the day of use, 0.1 ml of aqueous acetaldehyde (16 mg/ml) was added for each 20 ml of reagent needed. In estimating nucleic acids from biological sources, a suitable sample was obtained in 0.5 N $HClO_4$. A measured volume of 1 or 2 ml of sample (mix 1 ml sample with 1 ml 1 N $HClO_4$) was mixed with 2 ml of the diphenylamine reagent containing acetaldehyde, standards containing known amounts of DNA in 0.5 N $HClO_4$, and a reagent blank containing 0.5 N $HClO_4$, without DNA. The tubes were incubated overnight (16 to 20 h) at 30 C and the optical density at 600 nm was measured against a blank in a suitable spectrophotometer using 1 cm cells for comparison with values from the standard solutions. Beer's law was obeyed to 100 µg. The lowest amount measured was 5 µg (73).

Release of proline peptidase during autolysis of propionibacteria in a buffer system

Seventy-two-h-old cells of P. shermanii, P-59, and P. pentosaceum, P-9, grown in 200 ml of SLB, were isolated and washed as described earlier. The washed cells were resuspended in 40 ml of phosphate buffer (pH 6.8 and I = 0.3) and incubated at 32 C. Two 1-ml samples were taken from both suspensions and one of each was diluted to an optical density reading of 0.200 or less in deionized water, and the others in phosphate buffer (pH 6.8 and I = 0.3) for measurement of autolysis at 440 nm. Five-ml samples were removed from each of the solutions for measurement of proline iminopeptidase. The cells were removed by centrifugation at 16,000 x g for 10 min in a Sorvall refrigerated centrifuge at 0 C. The suspensions were sampled after 0, 1, 3, 6, 9, and 12 h. Proline and proline iminopeptidase released into the buffer were measured as described earlier.

RESULTS AND DISCUSSION

Survey of Commercial Swiss Cheese

Proline

As mentioned in the Introduction, proline is found in higher amounts in Swiss cheese than in any other cheese. If proline is important for the sweet flavor of Swiss cheese, as implied by some researchers (82, 241), addition of proline to fresh Swiss cheese, as removed from the warm room before full eye formation, should increase its sweetness. The amount of proline added should not exceed amounts found in a fully ripened Swiss cheese (200 to 600 mg proline/100 g cheese, Hintz et al. (82)). To test this hypothesis, we ground fresh cheese and divided it into three parts. To the first part, 250 mg proline/100 g cheese were added. To the second part, 500 mg were added. The third portion was held as a negative control. The samples were held overnight at 4 C so that proline would be dissolved and evenly distributed throughout the samples before they were submitted to a taste panel.

Information concerning the cheeses was not given to the panel before tasting. All members of the taste panel selected the cheese with 500 mg proline added/100 g cheese as the sweetest; most of the members (6 of 10) also found that the cheese sample with 250 mg proline/100 g cheese

was the second sweetest. The control cheese was considered as very bland with no apparent sweet flavor. The proline content in the control cheese was 65.84 mg/100 g cheese, far below the flavor threshold of proline [300 mg/100 g (97)]. The flavor threshold, as stated, was determined in water; other compounds such as fats, proteins, and volatile flavor compounds may influence the flavor threshold of proline in Swiss cheese. The taste panel also said that the flavor of the cheese with the highest proline content was the cheese with the best Swiss cheese flavor, even if it was rather bland. These results indicated that proline is important as a portion of Swiss cheese flavor, but other flavor compounds also are definitely important to produce full flavor.

To further substantiate this finding, we decided to submit commercial Swiss cheeses, both domestic and imported, to a taste panel, and asked the members to differentiate among the cheeses on the basis of sweetness. The taste panel also was asked to comment on the Swiss cheese flavor itself (if the flavor was balanced or unbalanced). The term "unbalanced" was used to describe a Swiss cheese flavor lacking the so-called nutty flavor. If the cheese was rancid, oxidized, or putrid, these terms were used because these flavors could overshadow natural Swiss cheese flavor. The cheeses were purchased locally in different stores or were

mailed to us from different cheese manufacturers. After the cheeses had been judged, they were sampled and analyzed for proline.

Quantitative results for the proline content of the cheeses and flavor descriptions are shown in Table 8. All the cheeses described as sweet, had a proline content higher than 300 mg/100 g cheese. If the proline content was less than 300 mg/100 g cheese and higher than 200 mg/100 g cheese, the description generally indicated that the cheeses contained some sweet flavor. Sweet flavor was not observed in cheeses with a proline content less than 200 mg/100 g cheese. All regular Swiss cheeses described as having a sweet, balanced flavor contained more than 300 mg proline/100 g cheese, Iowa-style Swiss (ISU) cheese described as having some sweet flavor and with a balanced total flavor, however, contained less than 300 mg proline/100 g cheese. These differences certainly could be caused by make procedures and block sizes.

All cheeses containing over 300 mg proline/100 g cheese, however, were not sweet. For example, an imported Swiss cheese containing 404 mg proline was described as having no sweet flavor, and was stale and oxidized. Other cheeses which contained high amounts of proline and were found to have some sweet flavor were often described as either rancid and/or fruity. These off-flavors may

Table 8. Proline content and flavor of Swiss cheeses

Cheese sample	Proline content mg/100 g	Flavor
Swiss, imported	834.50	Sweet, balanced flavor
Swiss, slices, domestic	722.00	Sweet, mild balanced flavor
Swiss, slices, domestic	697.63	Sweet, balanced flavor
Swiss, domestic	653.4	Sweet, bitter, balanced flavor
Swiss, slices, domestic	640.63	Sweet flavor, rancid
Swiss, slices, domestic	570.12	Some sweet, overshadowed by rancid, fruity flavors
Swiss, slices, imported	550.30	Sweet, mild balanced flavor
Swiss, domestic	542.70	Sweet, slightly bitter, balanced
Swiss, domestic	540.30	Sweet, mild, balanced flavor
Swiss, imported	536.46	Some sweet, unbalanced
Swiss, slices, domestic	534.86	Some sweet, balanced
Swiss, domestic	531.60	Sweet, balanced flavor
Swiss, imported	523.22	Some sweet flavor, balanced
Swiss, domestic	522.60	Sweet, balanced flavor
Swiss, domestic	487.50	Some sweet flavor, balanced
Swiss, domestic	424.40	Sweet, balanced bitter flavor

Table 8 (Continued)

Cheese sample	Proline content mg/100 g	Flavor
Swiss, domestic	424.0	Sweet, balanced flavor
Swiss, imported	404.32	Stale, oxidized flavor
Swiss, domestic	357.51	Some sweet flavor, rancid
Swiss, domestic	348.30	Sweet, balanced flavor
Swiss, domestic	340.40	Sweet, balanced flavor
Swiss, domestic	331.60	Some sweet flavor, fruity rancid
Swiss, domestic	324.03	Sweet, balanced flavor
Swiss, domestic	319.50	Sweet, balanced flavor
Swiss, domestic	316.30	Sweet, rancid, unbalanced flavor
Swiss, domestic	311.35	Some sweet flavor, rancid, unbalanced
Swiss, domestic	310.50	Some sweet flavor, rancid, bitter, unbalanced
Swiss, domestic	281.79	Weak, sweet flavor, rancid, bitter, unbalanced
Swiss, domestic	279.60	Weak, sweet flavor, bitter, unbalanced
Swiss, imported	273.32	No sweet flavor, unbalanced
Swiss, domestic (ISU)	265.30	Some sweet flavor, balanced
Swiss, domestic (ISU)	256.90	Some sweet flavor, balanced

Table 8 (Continued)

Cheese sample	Proline content mg/100 g	Flavor
Swiss, domestic	256.60	Some sweet flavor, rancid, slightly bitter, unbalanced
Swiss, domestic (ISU)	211.25	Some sweet flavor, balanced
Swiss, domestic	207.11	No sweet flavor, bland
Swiss, domestic	199.34	No sweet flavor, slightly acid
Swiss, imported (Jarlsberg)	186.52	Some sweet flavor, balanced
Swiss, domestic (ISU)	122.65	No sweet flavor, bland
Swiss, domestic (ISU)	116.47	No sweet flavor, bland
Swiss, domestic	84.71	No sweet flavor, putrid
Swiss, domestic	79.80	No sweet flavor, bland

possibly camouflage the sweet flavor. Some of the cheeses described as being unbalanced also had some rancid flavor. Most of these cheeses contained close to 300 mg proline. All regular Swiss cheeses with a proline content between 200 and 300 mg proline were unbalanced in flavor, while the Iowa-style Swiss cheeses had a balanced flavor at the same low proline content.

The results showed that proline content is related to

the sweet flavor of Swiss cheese as suggested by Virtanen and Kreula (241). The amount of proline necessary to give a sweet flavor was about 200 mg/100 g cheese confirming the results of Hintz et al. (82). A proline content over 200 mg/100 g Swiss cheese did not mean that the cheese had a balanced flavor, because the amount of other flavor compounds present also would influence the flavor. The sweet flavor probably does not come only from proline but, also, the other sweet amino acids, glycine, alanine, serine, and threonine may be important. Proline, or the other sweet amino acids, may not be the only compounds in Swiss cheese which are sweet, because an unknown, fat-soluble sweet compound from Swiss cheese has been observed (S. Biede, unpublished results).

Total free amino acids

All cheeses analyzed for proline, also were sampled and analyzed for total amounts of free amino acids by ion-exchange chromatography because free amino acids are assumed to be important for the background flavor of Swiss cheese. It was observed during the proline analyses that a high proline content did not necessarily indicate or predict a high-quality Swiss cheese. Because of the different descriptions of flavors and proline content categories, the cheeses were divided into seven groups;

1. Sweet, balanced flavor. Proline > 300 mg/100 g
2. Sweet, rancid flavor. Proline > 300 mg/100 g
3. Sweet, unbalanced flavor. Proline > 300 mg/100 g
4. Some sweet flavor, unbalanced. Proline content between 200 and 300 mg/100 g cheese.
5. No sweet flavor. Proline < 200 mg/100 g
6. Iowa-style Swiss cheese, balanced flavor. Proline < 300 mg/100 g cheese
7. Fresh Iowa-style Swiss cheese, no flavor. Proline < 200 mg/100 g cheese

This division was proposed because it was though that differences in the composition of the free amino acids might be related to cheese-flavor differences.

Also included in the analysis were five Italian cheeses. Both L. bulgaricus and S. thermophilus are used as starters for these cheeses, as in Swiss cheese, however, propionibacteria are added to Swiss cheese. Starter amounts used in Italian cheese manufacture are considerably higher than in Swiss cheese (188).

Calculation of the cheese free amino acids contents was done with an IBM, Model 360 computer, and it was decided to present the data in μMoles/100 g and in Mole percentage, because this would afford a better comparison between the different amino acids. The calculated values were presented as mean values and minimum and maximum values within each group. Results are presented in Tables A1 and A2 in the

Appendix. Data obtained through amino acid analysis were submitted to a statistical analysis. First, an analysis of variance was performed and the results were used to calculate t-values [Student's t-test (215)]. Swiss cheeses in the group of sweet, balanced cheeses were first compared against all other groups, and, afterwards, the group containing sweet, balanced Swiss cheeses was compared against the other Swiss cheese groups. The statistical analyses were performed on both absolute amounts (μMole/100 g) and relative amounts (Mole percentage).

Results of the statistical analysis of the absolute values and the sums of free amino acids are given in Table 9. The sums of the free amino acids can be regarded as a measurement of proteolysis, even though peptides are not included. The results show that proteolysis is more extensive in Italian cheeses than in the sweet, balanced Swiss cheeses. This higher level of proteolysis is probably caused by the higher amounts of high-temperature starters used in manufacture of the Italian cheeses. Cheese age also will have some influence. Total amounts of free amino acids in unbalanced cheeses, both in the sweet flavor group and the group with a low level of sweetness, were lower. This similarity is due to a lower degree of proteolysis even if the cheeses are of comparable age. A low-flavored, regular Swiss cheese showed definitely lower proteolysis. When regular, well-balanced flavor Swiss

Table 9. Statistical analysis of absolute values of free amino acids in different flavor groups of Swiss cheeses[a,b]

Treatment[c]	Number of observations	Means of amino acids (μMole/100 g)					
		Aspartic acid	Threonine	Serine	Asparagine	Glutamic acid	Glutamine
SB	21	66.22	975.99	916.45	857.55	5736.38	705.31
SR	5	74.54	897.49	923.76	1111.41	5212.38	596.66
SU	5	80.05	664.31**	713.33	966.26	4203.63**	510.51**
LU	4	39.32	706.31	680.35	913.24	4392.05	522.58
NO	3	26.52	400.96*	453.33**	733.08	2238.46*	324.79*
IS	4	59.85	477.40*	406.14*	732.30	2812.57*	506.61
IN	3	28.20	149.18*	110.05*	169.21*	1045.64*	130.79*
IT	5	727.74*	1481.60*	2653.36*	2125.60*	9282.60*	555.53

[a]Included in the analysis is a group of Italian cheeses.

[b]Statistical analysis included an analysis of variance between all groups of cheeses. The means and the error from this analysis were used to calculate the t-values by Student's t-test. t-values indicate level of significance.

[c]SB = sweet, balanced flavor Swiss cheese, SR = sweet, rancid Swiss cheese, SU = sweet, unbalanced flavor Swiss cheese, LU = low degree of sweetness, unbalanced flavor Swiss cheese, LO = low-flavored Swiss cheese, IS = Iowa-style Swiss cheese, balanced flavor, IN = Iowa-style Swiss cheese, no flavor, IT = Italian cheeses.

*Level of significance, 5% or better.

**Level of significance, 10%.

Table 9 (Continued)[a,b]

Treatment[c]	Proline	Glycine	Alanine	Valine	Methionine	Isoleucine	Leucine
SB	4207.94	1207.11	2104.22	2382.52	522.47	1233.25	4405.80
SR	3971.05	1124.60	2137.11	2341.51	570.56	1284.19	4407.05
SU	2443.33*	973.71	1517.47**	1735.30**	384.68**	718.57*	3530.57**
LU	2242.15*	958.50	1317.24*	1685.76**	403.86	537.16*	3540.12**
NO	1380.59*	498.39*	888.96*	899.26*	232.05*	413.99*	2259.49*
IS	1689.49*	556.88*	1003.11*	1165.19*	375.53**	385.70*	2692.68*
IN	726.44*	196.89*	496.07*	421.16*	148.32*	146.49*	1319.56*
IT	5032.62	1708.38*	1871.34	3185.82*	822.87*	2453.43*	5417.23**

Table 9 (Continued)[a,b]

	Tyrosine	Phenylalanine	γ-Amino Butyric acid	Ammonia + Ornithine	Lysine	Histidine	Arginine	Sum
SB	263.29	1479.61	52.93	2105.62	3085.86	822.37	21.42	33152.87
SR	297.98	1523.86	51.40	1951.89	3443.53	924.41	41.77	32887.11
SU	357.64	1090.64*	3.97	1471.62*	2153.09**	521.88*	11.18	24051.37*
LU	358.49	1023.23*	0.00	1281.05*	1744.65*	396.03*	4.09	22776.14*
NO	247.26	743.63*	4.98	941.74*	1303.33*	397.02*	36.48	14424.32*
IS	275.11	857.67*	19.99	962.79*	1643.94*	311.61*	264.33*	17198.84*
IN	134.57*	364.64*	0.00	290.98*	959.85*	143.62*	37.06	6518.69*
IT	686.69*	1928.25*	105.19	1323.45*	3712.45	966.25	n.d.	46061.38

cheeses were compared with well-balanced flavor Iowa-style Swiss cheeses, the degree of proteolysis was much lower in the Iowa-style Swiss cheeses. This difference in proteolysis is probably caused by different make procedures and different block sizes. Iowa-style Swiss is made in small blocks, and it is known that proteolysis is higher in large blocks (234).

Differences between individual amino acid content are easily observed between the balanced flavor, regular Swiss cheese and the Italian cheeses. Most of the free amino acid contents are significantly higher in Italian cheeses than in the balanced flavor Swiss cheeses except for glutamine, proline, alanine, γ-amino butyric acid, ammonia + ornithine, lysine, and histidine, with which the amounts were about the same or were even lower. Differences between the balanced and the unbalanced flavor Swiss cheeses were very small and only proline, alanine, isoleucine, leucine, phenylalanine, ammonia + ornithine, and histidine showed significantly lower values. These differences in absolute values may have some influence on the flavor of the cheeses. The variation between the balanced Swiss cheeses and the other flavor groups of Swiss cheeses with much lower degrees of proteolysis led to significant differences in essentially all amino acids. Iowa-style Swiss cheeses, made in smaller blocks, show about the same amounts of free amino acids as a small Gruyére cheese [Ritter et al. (190)], and both contained very high amounts

of arginine, which finding is in stark contrast to the here-in reported arginine content of regular Swiss cheese.

Another way to study the differences between free amino acids in different flavor groups of Swiss cheeses would be to study the relative amounts of amino acids present. Differences in relative amounts may easily be caused by differences in starter composition, more than differences in make procedures and block sizes. The results are shown in Table 10. First, a comparison was made between the balanced flavor Swiss cheeses and Italian cheeses. The Italian cheeses contained higher amounts of aspartic acid, threonine, serine, asparagine, glutamic acid, isoleucine, and tyrosine, and lower amounts of glutamine, proline, alanine, and ammonia + ornithine than the balanced flavor Swiss cheeses. These results fit with the observation of Antila (2) that propionibacteria could degrade aspartic acid, serine, glutamic acid, and tyrosine. This could explain part of the reason for the lower contents of these amino acids in Swiss cheese. Degradation of these amino acids leads to accumulation of large amounts of ammonia (1) which is found in higher amounts in Swiss cheese than in Italian cheeses. It has been indicated by Virtanen (239) that propionibacteria contain no proteolytic enzymes, but Berger et al. (12) found a tripeptidase in propionibacteria. This finding shows that propionibacteria probably will change the relative amounts of amino acids, and, therefore, will

Table 10. Statistical analysis of relative values of free amino acids in different flavor groups of Swiss cheeses[a,b]

Treatment[c]	Number of observations	Means of amino acids (Mole %)							
		Aspartic acid	Threonine	Serine	Asparagine	Glutamic acid	Glutamine	Proline	Glycine
SB	21	0.19	2.83	2.64	2.38	17.06	2.17	12.64	3.62
SR	5	0.22	2.74	2.83	3.32	15.91	1.77	11.90	3.40
SU	5	0.35**	2.73	2.95	4.25*	17.60	2.20	10.04*	4.06*
LU	4	0.17	3.03	2.94	4.09*	19.20*	2.31	9.83*	4.22*
NO	3	0.20	2.98	2.96	3.91**	15.82	1.98	8.68*	3.22
IS	4	0.35	2.74	2.30	4.03*	16.49	2.82*	10.16*	3.20
IN	3	0.45*	2.40	1.79*	2.78	16.31	2.16	11.45	3.03*
IT	5	1.56*	3.22**	5.78*	4.64*	20.13*	1.22	10.98*	3.71

[a]Included in the analysis is a group of Italian cheeses.

[b]Statistical analysis included an analysis of variance between all groups of cheeses. The means and the error from this analysis were used to calculate the t-values by Student's t-test. t-values indicate level of significance.

[c]SB = sweet, balanced flavor Swiss cheese, SR = sweet, rancid Swiss cheese, SU = sweet, unbalanced flavor Swiss cheese, LU = low degree of sweetness, unbalanced flavor Swiss cheese, LO = low-flavored Swiss cheese, IS = Iowa-style Swiss cheese, balanced flavor, IN = Iowa-style Swiss cheese, no flavor, IT = Italian cheeses.

*Level of significance, 5% or better.

**Level of significance, 10%.

Table 10 (Continued)

Treatment	Alanine	Valine	Methionine	Isoleucine	Leucine	Tyrosine	Phenyl-alanine	γ-Amino Butyric acid	Ammonia + Ornithine
SB	6.38	7.16	1.60	3.63	13.66	0.84	4.63	0.18	6.52
SR	6.32	6.94	1.73	3.98	13.46	0.94	4.61	0.17	6.07
SU	6.29	7.21	1.60	2.86*	14.83	1.48*	4.57	0.02	6.12
LU	5.77	7.45	1.76	2.33*	15.56**	1.55*	4.48	0.00	5.81
NO	6.36	6.96	1.77	2.94*	16.57**	1.38	5.48**	0.02	7.29
IS	5.69	6.72	2.19*	2.18*	15.74**	1.56*	4.98	0.11	5.56
IN	8.48*	6.36**	2.29*	2.26*	18.83*	2.01*	5.16	0.00	4.46*
IT	4.04*	6.90	1.78	5.33*	11.76	1.50*	4.18	0.23	2.84*

Table 10 (Continued)

Treatment	Lysine	Histidine	Arginine
SB	9.29	2.50	0.07
SR	10.65	2.88	0.14
SU	8.66	2.13	0.05
LU	7.59**	1.74*	0.16
NO	8.85	2.35	0.27
IS	9.46	1.89**	1.84*
IN	6.99*	2.16	0.61**
IT	8.10	2.11	n.d.

influence the amino acid composition of Swiss cheese by amino-acid degradation and peptide breakdown. Generally, in a cheese using S. thermophilus and/or L. bulgaricus as starter(s), the peptide content increased continuously during ripening of the cheese (180). Peptides will be precursors for peptide degradation by propionibacteria in Swiss cheese.

As mentioned earlier, proline content was related to the sweet flavor of Swiss cheese, and the Italian cheeses, which contained higher amounts of proline than the flavor threshold (97), also were found to be sweet, even though the total flavor was very different from that of Swiss cheese.

In the earlier comparison between the balanced Swiss cheeses and the unbalanced flavor Swiss cheeses, a significant difference was found between the sum of the free amino acids. The unbalanced cheeses contained proline in amounts close to the flavor threshold, and were found to be sweet, but the total flavor was unbalanced. This condition could be caused by quantitative and qualitative differences in many of the flavor compounds, but it was decided to determine if differences could be observed in the relative amounts of free amino acids in the cheeses. Statistical analysis showed that the unbalanced flavor cheeses contained higher amounts of asparagine, glutamic acid, glycine, and tyrosine, and lower amounts or proline, isoleucine, and histidine. These dif-

ferences may possibly be due to a lower degree of proteolysis, which has not released a high enough amount of peptides during the ripening of cheese. This may be one of the reasons why the amino acid composition was somewhere between an Italian cheese and a balanced flavor Swiss cheese. One observation worth mentioning was that the proline content was lower and the glycine content was higher in the unbalanced flavor cheeses than in the balanced flavor cheeses. Both proline and glycine are sweet in flavor.

Comparing the amino acid composition of regular Swiss cheeses with no sweet flavor and a very low degree of proteolysis to balanced flavor Swiss cheeses, the only significant difference in relative amounts is a lower proline and isoleucine content and a higher leucine content. This bland cheese was of the same age as the balanced flavor Swiss cheese so, obviously, something was wrong with the fermentation, even though the cheese had desirable eyes and texture.

A comparison between an Iowa-style Swiss cheese and a regular, balanced flavor Swiss cheese showed definite differences in proteolysis, which should somehow, affect the background flavor. The proline content of the former cheese was less than the proline flavor threshold, but the sum of all sweet amino acids; threonine, serine, proline, glycine, and alanine, would, of course, be high enough to give a sweet flavor. Asparagine, glutamine, methionine, tyrosine, and

arginine were found in higher amount in the Iowa-style Swiss, and proline, isoleucine, and histidine were in lower amounts. In Iowa-style Swiss cheese, S. lactis is used as a starter, in addition to the other three species, and may influence protein breakdown. Proteolysis also may be influenced by the different make procedure and block size. A fresh Iowa-style Swiss cheese contained definitely higher amounts of leucine and alanine than a balanced flavor Iowa-style Swiss cheese, and the amounts of both of these amino acids decreased during ripening.

Few other studies have been done on the free amino acid composition of Swiss cheese. The most recent study was made by Antila and Antila (3), in 1968. Their analyses showed a much lower amount of total free amino acids than was determined in this study. The content of glutamic acid in the Finnish Emmental cheeses they analyzed, also was much lower than reported in this study. Ritter et al. (190) studied the amino acid composition of small blocks of Gruyére cheeses, and their findings for 130-day-old cheeses showed some relationship to Iowa-style Swiss cheese. Otherwise, these analyses reported herein show large discrepancies from earlier Swiss cheese analyses.

Influence of groups and ratios of single amino acids and groups of amino acids

Usually, proline is the only amino acid which has been associated with the specific flavor of Swiss cheese. Other amino acids have only been related to background flavors. Because other amino acids have been reported to be sweet, it was decided to test the relationship between groups of amino acids within the different groups of cheeses. It also was decided to calculate ratios of certain amino acids and ratios of different groups, because it has been reported that the ratio of glutamic acid to proline (3) and the ratio of bitter amino acids to sweet amino acids (45) will differentiate between a poor-quality and a high-quality Swiss cheese.

The term "acids" was used to designate the acid amino acids; glutamic acid and aspartic acid, "basic" for the basic amino acids; lysine, histidine, and arginine, and "bases" for the basic amino acids plus ammonia and ornithine. Two separations made on the basis of the flavor of amino acids were based on information from the literature (97, 170). The sweet amino acids consisted of glycine, alanine, serine, threonine, and proline, all of which are neutral amino acids. The bitter amino acids are isoleucine, leucine, valine, phenylalanine, tyrosine, lysine, histidine, and arginine, which are neutral amino acids with either a long aliphatic or aromatic side chain, and basic amino acids.

Results are given in Tables A3, A4 and A5 in the Appendix. Data from statistical analysis of the absolute values are shown in Table 11. In the comparison between the balanced flavor Swiss cheeses and the Italian cheeses, the Italian cheeses contained significantly higher amounts of all groups except for the basic amino acids and the bases. Lower amounts of basic amino acids were produced in Italian cheeses. When the sweet balanced flavor Swiss cheeses were compared against the other Swiss flavor groups and Swiss-type cheeses, no significant differences were found with the sweet, rancid cheeses. However, except for the sweet, unbalanced and the low sweet, unbalanced flavor cheeses which contained quite high amounts of acid amino acids, other cheese groups contained significantly lower amounts than the balanced flavor Swiss cheeses.

When statistical analyses were performed on the relative values (Table 12) the statistical differences were few. Only the acid amino acids and the bases showed statistically significant differences between the Italian cheeses and the balanced flavor Swiss cheeses. The other flavor groups were comparable, even though significant statistical differences were found between a high number of the individual amino acids. Differences were found between the acid amino acids and the bases, and, also, differences between the sweet amino acids, but with lower significance, between the balanced Swiss

Table 11. Statistical analysis of absolute values of groups of amino acids in different groups of Swiss cheeses[a,b,c]

Treatment[d]	Number of observations	Means of groups of amino acids (μMole/100 g)				
		Acids	Basic	Bitter	Sweet	Bases
SB	25	5802.61	3929.64	13694.09	9412.31	6035.26
SR	5	5286.92	4409.71	14264.28	9054.00	6361.60
SU	5	4283.68**	2686.15*	10118.83*	6311.84*	4157.76*
LU	4	4431.37	2174.77*	9319.51*	5904.54*	3455.83*
NO	3	2267.98*	1736.83*	6300.47*	3622.24*	2678.58*
IS	4	2872.42*	2219.87*	7596.20*	4133.02*	3182.66*
IN	3	1073.83*	640.52*	3026.93*	1678.62*	931.51*
IT	5	10010.33*	4678.70	18350.11*	12768.29*	6002.15

[a]Included in the analysis is a group of Italian cheeses.

[b]Statistical analysis included an analysis of variance between all groups of cheeses. The means and the error from this analysis were used to calculate the t-values by Student's t-test. t-values gave the level of significance.

[c]Acids = Aspartic and glutamic acid. Basic = Lysine, histidine and arginine. Bases = Basic and ammonia + ornithine. Sweet = Glycine, alanine, serine, threonine, and proline, Bitter = Isoleucine, leucine, valine, phenylalanine, tyrosine, lysine, histidine, and arginine.

[d]SB = sweet, balanced flavor Swiss cheese, SR = sweet, rancid Swiss cheese, SU = sweet, unbalanced flavor Swiss cheese, LU = low degree of sweetness, unbalanced flavor Swiss cheese, LO = low-flavored Swiss cheese, IS = Iowa-style Swiss cheese, balanced flavor, IN = Iowa-style Swiss cheese, no flavor, IT = Italian cheeses.

*Level of significance, 5% or better.

**Level of significance, 10%.

Table 12. Statistical analysis of relative values of groups of amino acids in different groups of Swiss cheeses[a,b,c]

Treatment[d]	Number of observations	Means of groups of amino acids (Mole %)				
		Acids	Basic	Bitter	Sweet	Bases
SB	25	17.25	11.87	41.79	28.12	18.38
SR	5	16.10	13.67	43.61	27.20	19.73
SU	5	17.95	10.83	41.78	26.07**	16.96
LU	4	19.37*	9.48**	40.86	25.79**	15.29*
NO	3	16.02	11.47	44.81	24.19*	18.77
IS	4	16.84	13.18	44.36	24.09*	18.74
IN	3	16.76	9.77	44.39	27.16	14.22*
IT	5	21.69*	10.21	39.89	27.73	13.04*

[a]Included in the analysis is a group of Italian cheeses.

[b]Statistical analysis included an analysis of variance between all groups of cheeses. The means and the error from this analysis were used to calculate the t-values by Student's t-test. t-values gave the level of significance.

[c]Acids = Aspartic and glutamic acid. Basic = Lysine, histidine and arginine. Bases = Basic and ammonia + ornithine. Sweet = Glycine, alanine, serine, threonine, and proline, Bitter = Isoleucine, leucine, valine, phenylalanine, tyrosine, lysine, histidine, and arginine.

[d]SB = sweet, balanced flavor Swiss cheese, SR = sweet, rancid Swiss cheese, SU = sweet, unbalanced flavor Swiss cheese, LU = low degree of sweetness, unbalanced flavor Swiss cheese, LO = low-flavored Swiss cheese, IS = Iowa-style Swiss cheese, balanced flavor, IN = Iowa-style Swiss cheese, no flavor, IT = Italian cheeses.

*Level of significance, 5% or better.

**Level of significance, 10%.

cheese and the unbalanced flavor Swiss cheeses with a low degree of sweetness. The Iowa-style Swiss cheeses contained lower amounts of sweet amino acids than the balanced flavor Swiss cheeses. The results indicated that the total relative amounts of the different groups of amino acids, do not vary much between cheeses of different quality.

Table 13 presents the data from the statistical analysis of ratios of different groups of amino acids. Significant differences were found for the ratio of acid amino acids and basic aminc acids, and acid amino acids and bases, between balanced flavor Swiss cheeses and Italian cheeses. The Swiss cheese contains higher amounts of basic amino acids and ammonia than the Italian cheeses, and, also, somewhat lower amounts of acid amino acids. It is known that propionibacteria produce high amounts of ammonia during growth (1). Significant differences also were found between the ratios of sweet amino acids to acid amino acids. Comparing the ratio of bitter to sweet amino acids does not show a significant difference, not even between the sweet, balanced flavor Swiss cheeses and the two unbalanced flavor groups. This finding contradicts the report by Dylanyan et al. (45), that high-quality Swiss cheese has a lower ratio between bitter and sweet amino acids than low-quality Swiss cheese. The ratio was a little higher for the unbalanced flavor cheeses, but was not statistically significant. However, the difference was significant between

Table 13. Statistical analysis of ratios between groups of amino acids in different flavor groups of Swiss cheeses[a,b,c]

Treatment[d]	Number of observations	Means of ratios			
		Acids / Basic	Bitter / Sweet	Acid / Bases	Sweet / Acids
SB	25	1.54	1.51	0.98	1.64
SR	5	1.19	1.61	0.82	1.69
SU	5	1.731.	1.61	1.09	1.47**
LU	4	2.08*	1.59	1.29*	1.34*
NO	3	1.44	1.88*	0.87	1.52
IS	4	1.28	1.85*	0.91	1.44*
IN	3	1.72	1.77	1.18**	1.60
IT	5	2.34*	1.44	1.79*	1.28*

[a]Included in the analysis is a group of Italian cheeses.

[b]Statistical analysis included an analysis of variance between all groups of cheeses. The means and the error from this analysis were used to calculate the t-values by Student's t-test. t-values gave the level of significance.

[c]Acids = Aspartic and glutamic acid. Basic = Lysine, histidine and arginine. Bases = Basic and ammonia + ornithine. Sweet = Glycine, alanine, serine, threonine, and proline, Bitter = Isoleucine, leucine, valine, phenylalanine, tyrosine, lysine, histidine, and arginine.

[d]SB = sweet, balanced flavor Swiss cheese, SR = sweet, rancid Swiss cheese, SU = sweet, unbalanced flavor Swiss cheese, LU = low degree of sweetness, unbalanced flavor Swiss cheese, LO = low-flavored Swiss cheese, IS = Iowa-style Swiss cheese, balanced flavor, IN = Iowa-style Swiss cheese, no flavor, IT = Italian cheeses.

*Level of significance, 5% or better.

**Level of significance, 10%.

the balanced flavor Swiss cheeses and the Iowa-style Swiss cheeses, with the latter group containing higher amounts of bitter amino acids. It was, however, observed that the difference between sweet, balanced Swiss cheeses and unbalanced flavor Swiss cheeses with a low degree of sweetness was significantly different when looking at the ratios of acid amino acids to basic amino acids and to bases and the ratio of sweet amino acids to acid amino acids. Generally, the ratios of the unbalanced flavor, sweet cheeses were somewhat closer to those of the balanced flavor Swiss cheeses, but the values were between the Italian cheeses and the sweet balanced flavor Swiss cheeses. This may indicate a degree of proteolysis somehow closer to the use of high-temperature starters, and that the changes by propionibacteria in the cheese were less. The ratio of acid amino acids to bases and the ratio of sweet amino acids to acid amino acids may be indicative of quality for regular Swiss cheeses but do not serve for Iowa-style Swiss.

Comparing the ratios between single amino acids (Table 14), the ratio of glutamic acid to proline, leucine to isoleucine, proline to glycine, and phenylalanine to tyrosine describes differences between high-quality Swiss cheese and unbalanced flavor Swiss cheese. Balanced flavor cheeses contain lower amounts of glutamic acid, glycine, and tyrosine and

Table 14. Statistical analysis of ratios between selected amino acids from different groups of Swiss cheeses[a,b]

Treatment[c]	Number of observations	Means of ratios				
		Glutamic acid Proline	Threonine Serine	Leucine Isoleucine	Proline Glycine	Tyrosine
SB	25	1.35	1.10	3.93	3.52	10.55
SR	5	1.34	0.97	3.40	3.50	11.21
SU	5	1.82*	0.94**	5.60*	2.57*	3.19**
LU	4	2.03*	1.02	6.72*	2.42*	2.99**
NO	3	1.98*	1.05	5.79*	2.67*	12.98
IS	4	1.66**	1.21	7.36*	3.22	3.35**
IN	3	1.43	1.37*	8.39*	3.78	2.49*
IT	5	1.84*	0.56*	2.20*	2.96*	2.81*

[a]Included in the analysis is a group of Italian cheeses.

[b]Statistical analysis included an analysis of variance between all groups of cheeses. The means and the error from this analysis were used to calculate the t-values by Student's t-test. t-values indicated significance level.

[c]SB = sweet, balanced flavor Swiss cheese, SR = sweet, rancid Swiss cheese, SU = sweet, unbalanced flavor Swiss cheese, LU = low degree of sweetness, unbalanced flavor Swiss cheese, LO = low-flavored Swiss cheese, IS = Iowa-style Swiss cheese, balanced flavor, IN = Iowa-style Swiss cheese, no flavor, IT = Italian cheeses.

*Level of significance, 5% or better.

**Level of significance, 10%.

higher amounts of proline and isoleucine. Iowa-style Swiss cheeses were not significantly different except in the ratio between leucine and isoleucine, and contain significantly higher relative amounts of leucine than the balanced, regular Swiss cheeses. All ratios were significantly different between the balanced flavor Swiss cheeses and the Italian cheeses.

This analysis between different types and qualities of Swiss cheeses, shows that differences in amino acid composition and ratios of different amino acids and groups of amino acids will describe flavor quality differences. Part of the difference was due to different degrees of proteolysis, but, besides differences in absolute amounts, large differences in relative amounts also were observed. These variances may be the cause of flavor differences between an unbalanced and a balanced flavor Swiss cheese, but, because, they may also arise from an unbalanced fermentation, other flavor components may be involved. Therefore, the amounts of volatile fatty acids in the various cheeses also were considered.

Volatile free fatty acids, their ratios, and the ratio of propionic acid to proline in Swiss cheese of various flavor groups

Flavor development in Swiss cheeses depends partly on the relative amounts of volatile, free fatty acids (119), present in the cheeses. Therefore, the cheese samples in this

study were analyzed for free fatty acids in addition to free amino acids. The results were calculated in μMole/100 g, and the calculated values were presented as mean values as well as the minimum and maximum values within each group of cheeses. Results are presented in Table A6 in the Appendix. Data obtained through volatile fatty acid analyses were submitted to statistical analysis and the results are presented in Table 15. The Italian cheese data were not analyzed statistically.

The highest amounts of volatile fatty acids were produced in the sweet, balanced flavor Swiss cheeses and in the rancid cheeses. The butyric acid content was, as expected, significantly different between cheeses, being found in higher amounts in the sweet, rancid cheeses. Another fact worth mentioning was that, in a few samples of balanced flavor cheeses, the amount of butyric acid was at the same level as in the rancid cheeses, but the amounts of acetic and propionic acid were definitely higher in the balanced flavor cheeses. The difference in relative amounts of acetic and propionic acids may be of importance in obtaining impression of rancid flavor; that is, higher amounts of acetic acid propionic acid may mask the rancid flavor of butyric acid. Another possibility could be that the content of free higher fatty acids is present in higher amounts in sweet, rancid Swiss cheeses (119).

Table 15. Statistical analysis of volatile fatty acids, the ratio of propionic to acetic acid, and of propionic acid to proline[a]

Treatment[b]	Number of observations	Mean values of volatile fatty acids (μMole/100 g)			Mean values of ratios	
		Acetic acid	Propionic acid	Butyric acid	Propionic acid / Acetic acid	Propionic acid / Proline
SB	18	3997.0	4036.4	466.5	1.0222	1.0789
SR	5	3873.7	4706.4	649.5*	1.2200**	1.3500
SU	4	2908.9*	2711.4*	603.6**	0.9300	1.1575
LU	4	2690.6*	2596.3*	634.4**	0.9725	1.1900
LO	3	2683.8*	3317.3	526.0	1.1733	6.2167*
IS	4	2529.1*	3979.0	313.5	1.6350*	2.2725*
IN	3	1624.8*	2192.2*	296.0**	1.3900*	2.9967*

Statistical analysis included an analysis of variance between all groups of cheeses. The means and the error from this analysis were used to calculate the t-values by Student's t-test. t-values indicate significance level.

SB = sweet, balanced flavor Swiss cheese, SR = sweet, rancid Swiss cheese, SU = sweet, unbalanced flavor Swiss cheese, LU = low degree of sweetness, unbalanced flavor Swiss cheese, LO = low-flavored Swiss cheese, IS = Iowa-style Swiss cheese, balanced flavor, IN = Iowa-style Swiss cheese, no flavor.

*Level of significance, 5% or better.

**Level of significance, 10%.

When the groups of unbalanced flavor Swiss cheeses were compared with the balanced flavor Swiss cheeses, significantly lower amounts of acetic and propionic acid and higher amounts of butyric acid were found in the unbalanced flavor cheeses. The high amount of butyric acid present, of course, was the reason why most of these cheeses also were described as rancid in addition to the description "unbalanced." The lower amounts of acetic and propionic acid present also constituted a significant difference from the balanced flavor cheeses.

The main difference between Iowa-style Swiss cheeses and the sweet, balanced flavor Swiss cheeses was that the acetic acid content was definitely lower in the Iowa-style cheeses. Another fact which has been stated to be important for flavor is the ratio of propionic to acetic acid (122). Only between sweet, balanced flavor Swiss cheeses and Iowa-style Swiss cheeses were significant differences found in these ratios and both groups were high-quality cheeses. The ratios were determined on a molecular basis in this study, but in earlier studies the ratios were compared on a weight basis. By recalculation, the ratio was about 1.17 for the regular Swiss cheeses on a weight basis, which was just a little higher than information in the literature (117). For Iowa-style Swiss cheeses, the ratio of propionic to acetic acid was 2.0, which is comparable to that given in earlier results (135).

The difference was probably caused by different make procedures, which would influence the growth of propionibacteria in the cheese. It also is known that this ratio is about 2.0 during the first part of the growth period of propionibacteria in SLB, but that the ratio then changes later to about 1.0 (122).

Also, the ratio of propionic acid to proline has been suggested as describing quality differences in Swiss cheeses (122). However, the only significant difference in this respect was found between the sweet, balanced flavor Swiss cheeses and Iowa-style Swiss cheeses, both cheeses of high quality. Significant difference also was found for the low-flavored Swiss cheeses in comparison with the sweet, balanced flavor Swiss cheese, however, this difference was caused by one cheese containing extreme low amounts of proline.

Conclusion

Proline was found to be important for the sweet flavor of Swiss cheese. Swiss cheeses described as "sweet" contained more than 300 mg/100 g cheese. If a noticeable level of sweet flavor was observed, the proline content was between 200 and 300 mg/100 g cheese, and if no sweet flavor was detected, the proline content was less than 200 mg/100 g cheese. However, a high proline content did not necessarily signify high-quality cheese.

The cheeses were divided into groups on the bases of quality and types. Significantly higher amounts of free amino acids and free fatty acids were found in sweet, balanced flavor Swiss cheeses in comparison with the unbalanced flavor Swiss cheeses. The ratio of propionic to acetic acid was not different, but the content of butyric acid was higher in the unbalanced flavor cheeses. Large differences were found when relative amounts of free amino acids were compared between these flavor groups cheeses. The balanced flavor Swiss cheeses contained higher amounts of proline, isoleucine, and histidine, and lower amounts of asparagine, glutamic acid, glycine, and tyrosine. It also was found that the group of basic amino acids plus ammonia and ornithine were higher in the balanced flavor cheeses. The ratio of glutamic acid to proline was important in describing differences between the sweet, balanced flavor Swiss cheeses and the unbalanced flavor cheeses. Relative amounts of amino acids, therefore, are related to flavor quality differences, but it must be remembered that the low quality cheeses also contained lower amounts of free amino acids and fatty acids, both of which influence flavor. In regard to relative amounts of amino acids, the unbalanced flavor Swiss cheeses lie between the Italian cheeses and the balanced flavor Swiss cheeses. This may indicate the influence of propionibacteria on the amino

acid composition, and, specifically, on proline content. However, a complex relationship must exist between proline and the other flavor components which give a balanced Swiss cheese flavor. This relationship needs further study.

Proline Production by Propionibacteria

Proline production by propionibacteria in milk and fortified milk

The analysis of various Swiss cheeses strongly indicated that proline is an important factor in the sweet flavor of Swiss cheese. Because the relative amounts of proline were higher in balanced flavor Swiss cheese than in Italian cheese, it is a possibility that propionibacteria may be involved in production of proline in Swiss cheese. To prove this belief, it would be desirable to see if propionibacteria could produce proline in pure culture. To this end, P. shermanii, P-59, was grown in milk. Because milk is a poor substrate for propionibacteria (80, 150), it was fortified with 0.1% Trypticase, 0.1% yeast extract, or an equal mixture of both compounds. Lactose, the natural carbohydrate of milk, is not a good energy source for propionibacteria, so 0.1% glucose or 0.1% sodium lactate also were added to the milk medium.

The following media combinations were used:

1. Reconstituted milk

2. Reconstituted milk and 1 g/l Trypticase
3. Reconstituted milk and 1 g/l yeast extract
4. Reconstituted milk, 1 g/l Trypticase, and 1 g/l yeast extract
5. Reconstituted milk and 1 g/l glucose
6. Reconstituted milk, 1 g/l glucose, and 1 g/l Trypticase
7. Reconstituted milk, 1 g/l glucose, and 1 g/l yeast extract
8. Reconstituted milk, 1 g/l glucose, 1 g/l Trypticase, and 1 g/l yeast extract
9. Reconstituted milk and 1 g/l sodium lactate
10. Reconstituted milk, 1 g/l sodium lactate, and 1 g/l Trypticase
11. Reconstituted milk, 1 g/l sodium lactate, and 1 g/l yeast extract
12. Reconstituted milk, 1 g/l sodium lactate, 1 g/l Trypticase, and 1 g/l yeast extract

Results of these trials are shown in Figures 2, 3, and 4. With lactose as an energy source, the maximum cell number was found after 3 days; addition of Trypticase and yeast extract increased the total cell number, highest number being found in the milk containing both additives. Addition of glucose did not influence the growth. The cell number after 3 days was about the same, but, in comparison with milk, the numbers continued to increase slowly up to the 6th day. The difference between the control with glucose and those to which nitrogen compounds had been added was small. Sodium

Figure 2. Proline production and cell numbers of P. shermanii, P-59, at 32 C in reconstituted and fortified milk. (——) proline production, (----) cell numbers. Symbols: ● , no addition, o , 0.1% Trypticase, Δ , 0.1% yeast extract, □ , 0.1% Trypticase, 0.1% yeast extract

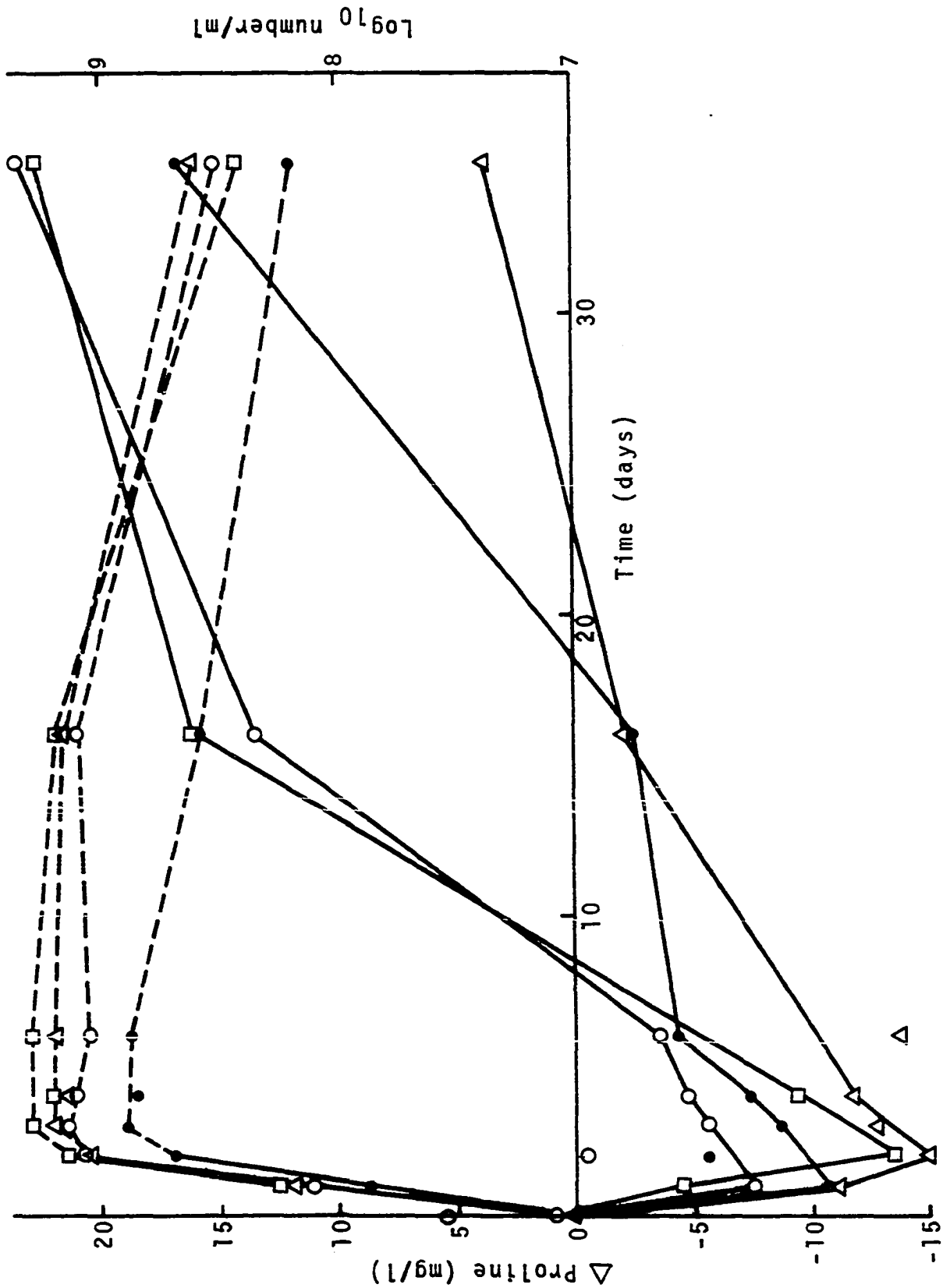

Log10 number/ml
9
8
7
Time (days)
30
20
10
0
Δ Protine (mg/l)
20
15
10
5
0
-5
-10
-15

Figure 3. Proline production and cell numbers of P. shermanii, P-59, at 32 C in fortified, reconstituted milk. (——) Proline production, (----) cell numbers. Symbols: ◇ , 0.1% glucose, o , 0.1% glucose and 0.1% Trypticase, △ , 0.1% glucose and 0.1% yeast extract, □ , 0.1% glucose, 0.1% Trypticase and 0.1% yeast extract

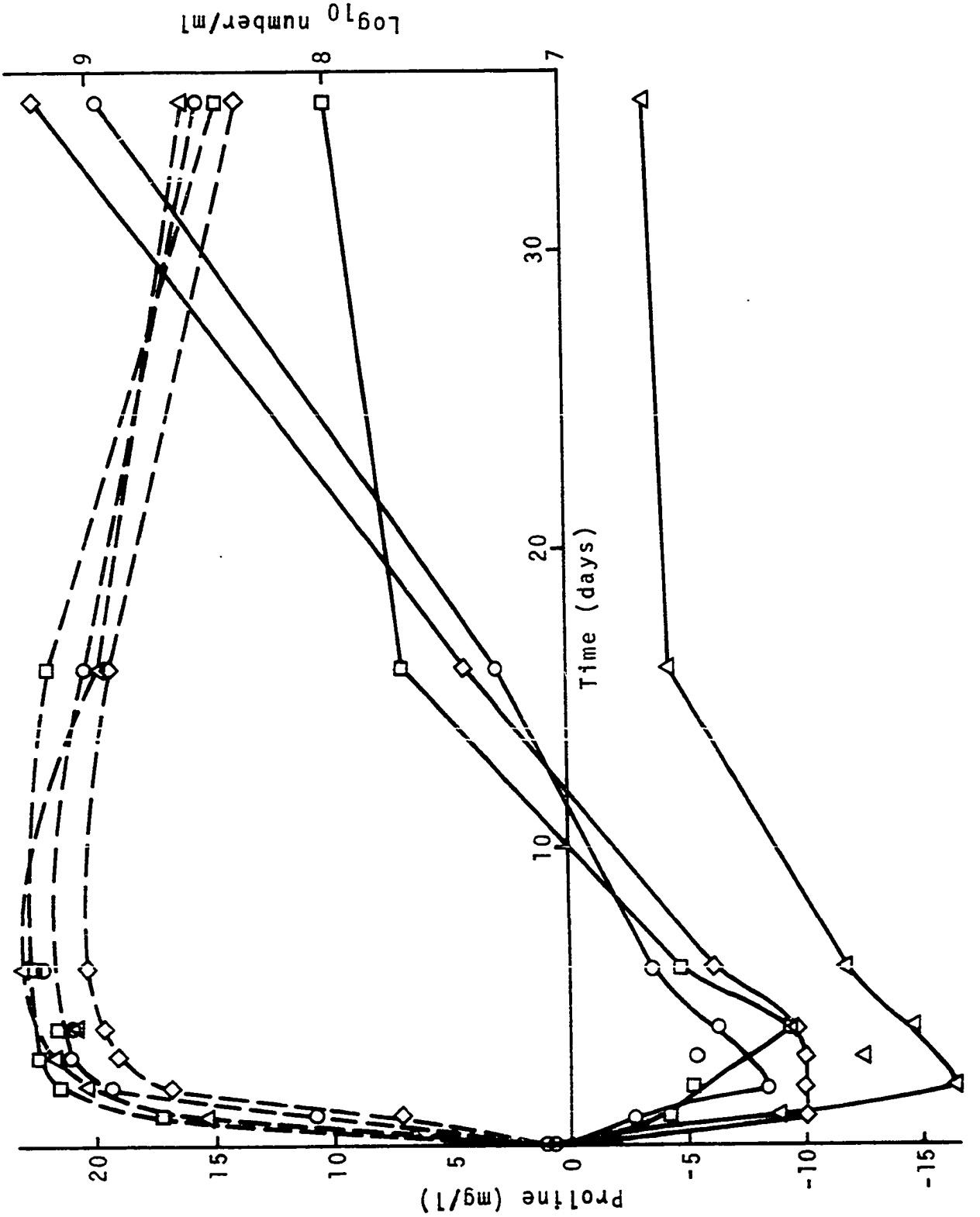
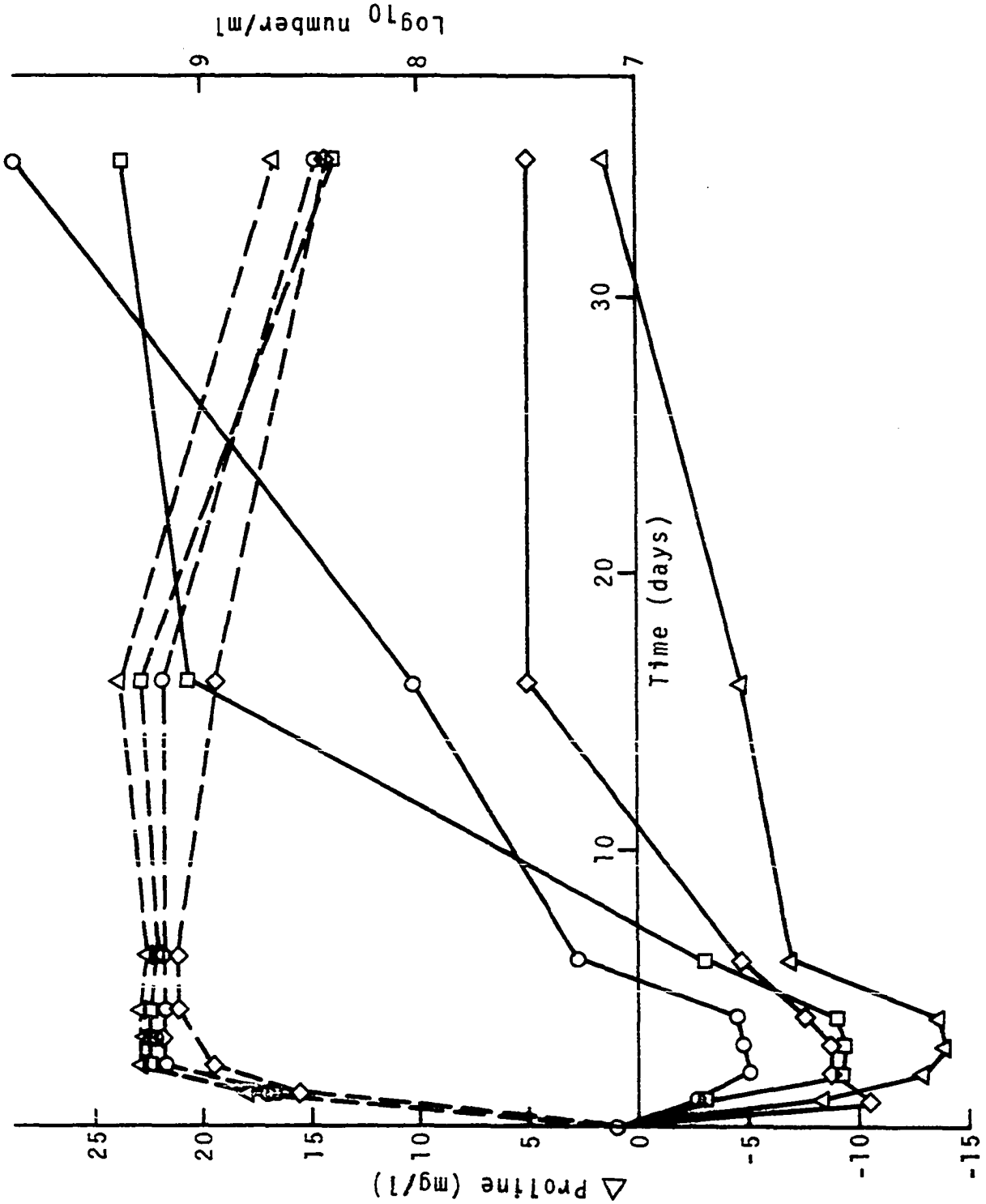

Log10 number/ml
9
8
7
30
20
10
Time (days)
Proline (mg/l)
20
15
10
5
0
-5
-10
-15

Figure 4. Proline production and cell numbers of P. shermanii, P-59, at 32 C in fortified, reconstituted milk. (——) proline production, (----) cell numbers. Symbols: ◇ , 0.1% sodium lactate, ○ , 0.1% sodium lactate and 0.1% Trypticase, △ , 0.1% sodium lactate and 0.1% yeast extract, □ , 0.1% sodium lactate, 0.1% Trypticase and 0.1% yeast extract


Log10 number/ml
6
8
7
Time (days)
30
20
10
Δ Protine (mg/l)
25
20
15
10
5
0
-5
-10
-15

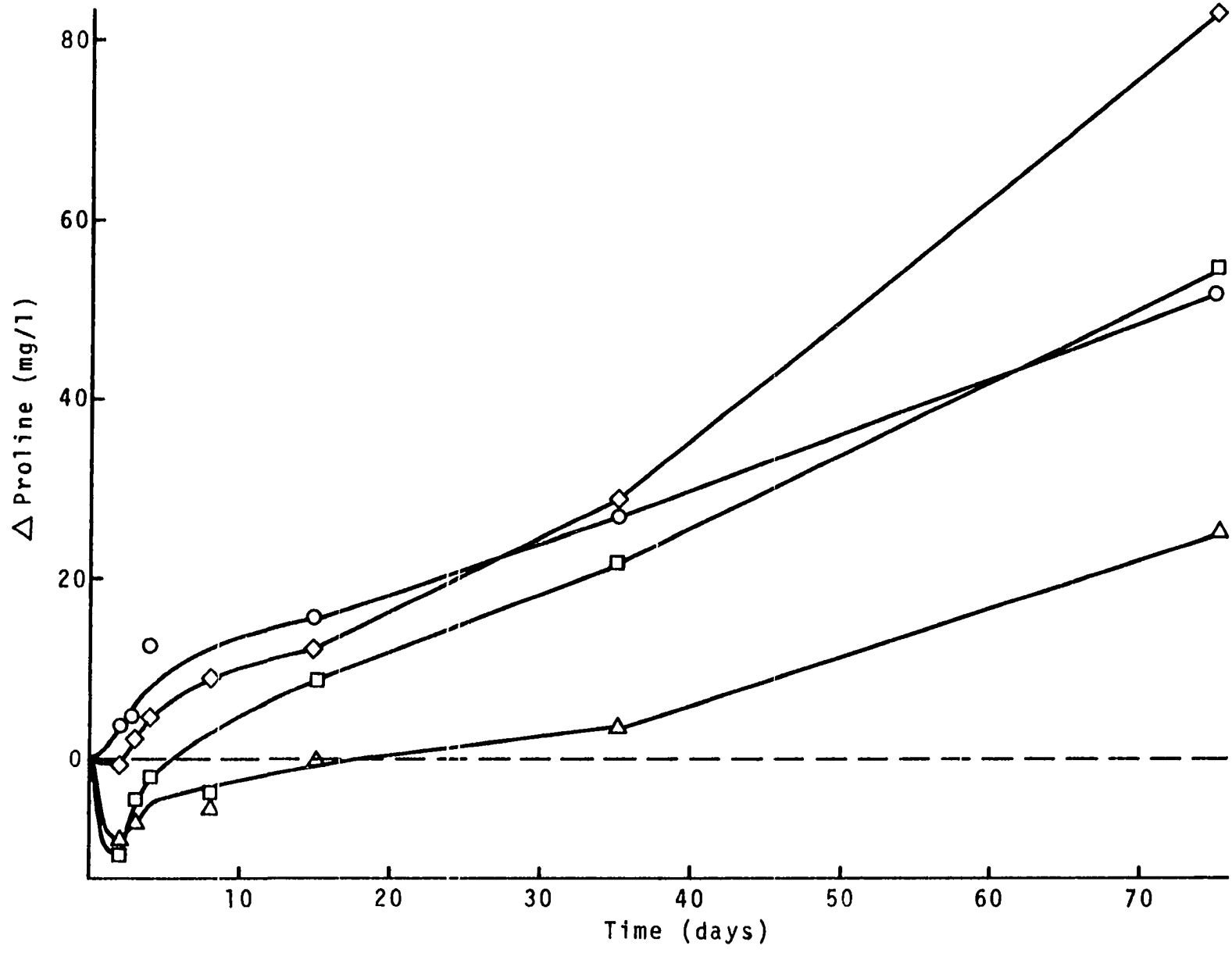
lactate addition increased total cell numbers in comparison with milk and milk with glucose, but addition of nitrogen compounds in addition to sodium lactate increased the numbers slightly in comparison with the sodium lactate control.

The free proline content of the milk medium is low, being only about 13 mg/l. By addition of Trypticase and yeast extract, the total free proline content was increased by, respectively, 17 and 12 mg/l. During the first 2 days proline was not produced, the amounts of free proline (in contrast) decreased. The least decrease occurred in the sample to which Trypticase was added, followed by the control with no added nitrogen source, and, then, the sample with both Trypticase and yeast extract. The largest decrease in proline took place in the medium to which yeast extract had been added (about 15 mg/l). This decrease took place during the logarithmic growth phase. After the 2nd day the proline content of the milk media started to increase. The highest amount of proline was produced in the medium with added sodium lactate, followed by the milk alone, and the least increase was produced in the milk with added glucose. These results were correlated with coagulation times, and therefore, with acid production by P. shermanii strain. In all cases the three media with yeast extract added alone to the milk, to milk with glucose, and to milk with sodium lactate

coagulated first; with added glucose after 12 days, with milk after 15 days, and with sodium lactate after 17 days. The amount of proline produced also was the lowest. Media with added glucose showed the slowest and lowest level of proline production. The media with added glucose, however, coagulated faster than the other media. The media with both Trypticase and yeast extract coagulated after 13 days, followed by the two other media after 16 days. With the unfortified milk, coagulation occurred after 16 days, followed by the milk containing Trypticase, and the mixture of Trypticase and yeast extract after 20 days. The most rapid proline production took place during the first 16 days. With the addition of sodium lactate, the control coagulated after 18 days, and the next to lowest amount of proline was produced. The most rapid proline production took place in the medium with added Trypticase and yeast extract for the first 16 days, but after 35 days the most proline was produced with the addition of Trypticase. The latter samples both coagulated after 24 days.

The last experiment mentioned was repeated to determine if proline would continue to increase after 35 days. Continuous proline production took place during the entire time period of 75 days (Figure 5). After 75 days, however, the most proline had been produced from the control medium.

Figure 5. Proline production by P. shermanii, P-59, at 32 C in fortified, reconstituted milk. Symbols: ◇ , 0.1% sodium lactate, o , 0.1% sodium lactate and 0.1% Trypticase, △ , 0.1% sodium lactate and 0.1% yeast extract, □ , 0.1% sodium lactate, 0.1% Trypticase and 0.1% yeast extract


80
60
40
20
0
Δ Proline (mg/l)
10
20
30
40
50
60
70
Time (days)

Addition of Trypticase, and Trypticase and yeast extract produced lesser amounts but still yielded much larger quantities than in the medium to which yeast extract had been added.

These results confirmed the earlier observation that milk is not a suitable medium for propionibacteria (80, 150). Addition of glucose did not lead to faster growth, but to earlier coagulation of the milk. Sodium lactate addition enhanced cell number, indicating that sodium lactate is a better energy source. The addition of Trypticase and yeast extract encouraged higher cell numbers. One of the limitations for rapid growth of propionibacteria would be the low amounts of amino acids and peptides found in milk (29, 141) as propionibacteria are not able to break down milk proteins (239). Hietaranta and Antila (80) also observed this effect and noted that addition of a cell-free culture filtrate from L. helveticus and S. thermophilus stimulated growth of propionibacteria. In Swiss cheese, these lactic-acid bacteria grow before the propionibacteria and produce better conditions for growth of propionibacteria both in regard to creating a better energy source and freeing higher amounts of peptides and amino acids than would be found in the uncultured milk.

In regard to proline production, rather small amounts were produced in the milk media, in amounts substantially smaller than the amounts found in Swiss cheese. Addition of

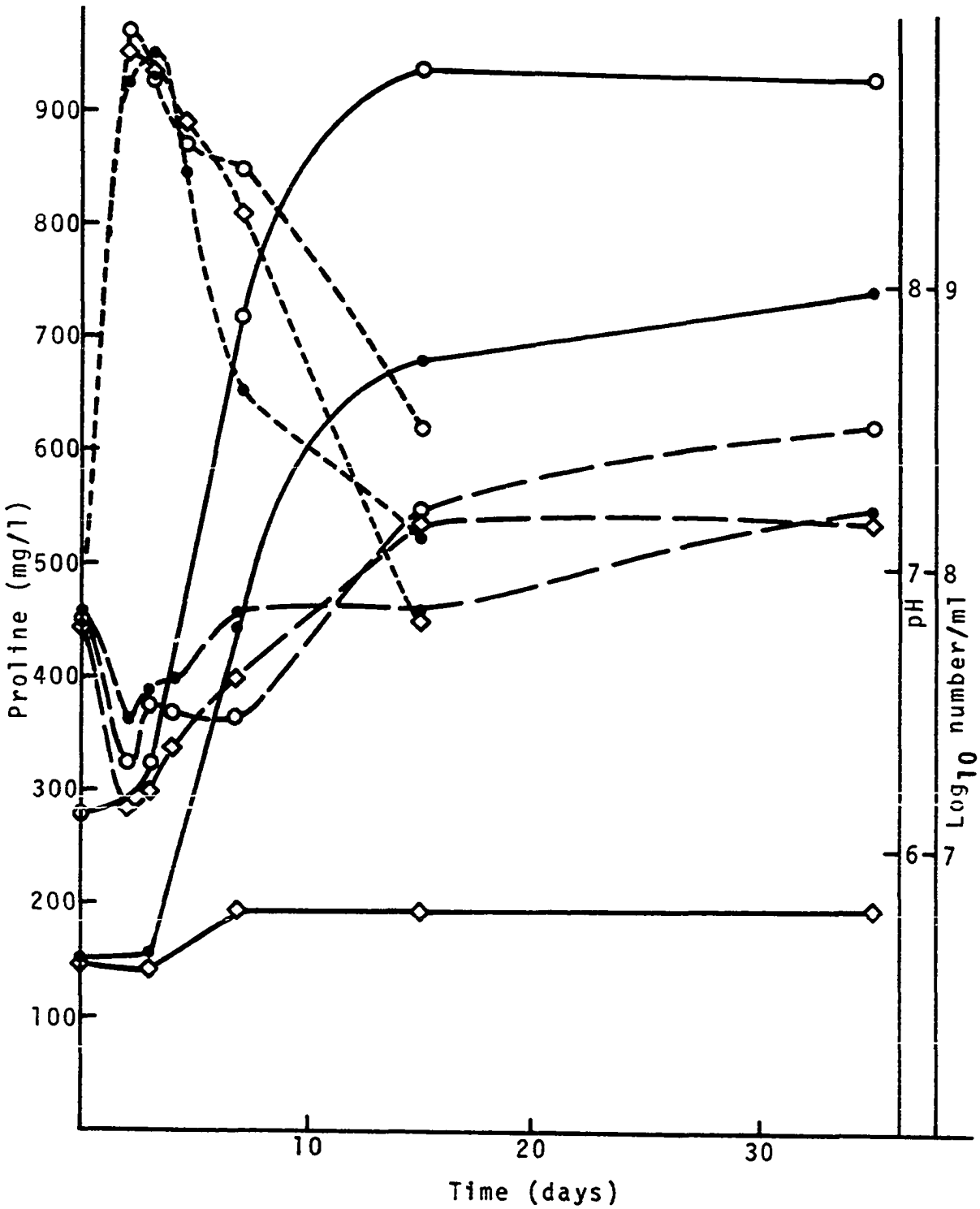
Trypticase, however, led to an increase in the rate of proline production during the first 16 days, and higher amounts of proline than in the control medium were most often produced. Inconsistencies were observed, which were most probably related to differences in coagulation time. Proline could possibly be produced by biosynthesis, and peptidase and protease activities as shown in Figure 1. Biosynthesis, however, did not seem probable because, from information in the literature, proline production seemed to follow the growth curve, or may be a little slower, as reported for mutants of Kurthia catenaforma and Brevibacterium flavum (86, 258). Substrate limitations, of course, may cause the slow proline production in the early growth stages, but free glutamic acid constitutes about 39% of the free amino acids in milk (141), and, besides, proline may be produced from lactose through pyruvate to α-ketoglutarate (124). The increased rate of proline production induced by Trypticase addition indicates that peptidases may be important, and the slower production at later stages, seems to imply intracellular proteolytic enzymes may be involved in proline liberation. The three mechanisms for proline production, however, may still be in effect, because conditions for maximum proline production probably are not present in unaltered milk.

Proline production in media containing Trypticase and yeast extract

The indication garnered from the last experiment that peptide addition increases proline production, and information from the literature (122) that the high-temperature starter organisms break down the cheese casein producing peptides and amino acids, implies that the next experiment, following biological order, should be to follow proline production in media containing high amounts of peptides. The usual growth medium for propionibacteria, SLB, was chosen. This medium, however, contained yeast extract and Trypticase; therefore, Trypticase was removed to produce a control medium. Another medium, in which the yeast extract had been exchanged with a defined vitamin solution, also was used to determine the effect of yeast extract on proline production from Trypticase.

Figure 6 gives proline production, total cell numbers, and pH changes in the different media as produced by P. shermanii, P-59. Proline content was not corrected for the initial amount in the media. Maximum cell numbers were reached after 2 days in SLB with or without Trypticase, and after 3 days in the medium with Trypticase but without yeast extract. Growth was faster and total cell numbers were higher than in milk. Measured at the same time during the logarithmic growth phase, pH values dropped from pH 6.8 to

Figure 6. The relationship between total free proline, viable cell numbers, and pH changes in three different media during growth of P. shermanii, P-59, at 32 C. (———), total free proline, (----), pH changes, (····), viable cell numbers. Symbols: ◇ , Control, SLB without Trypticase, o, SLB, ● , SLB in which yeast extract has been exchanged with a defined vitamin solution

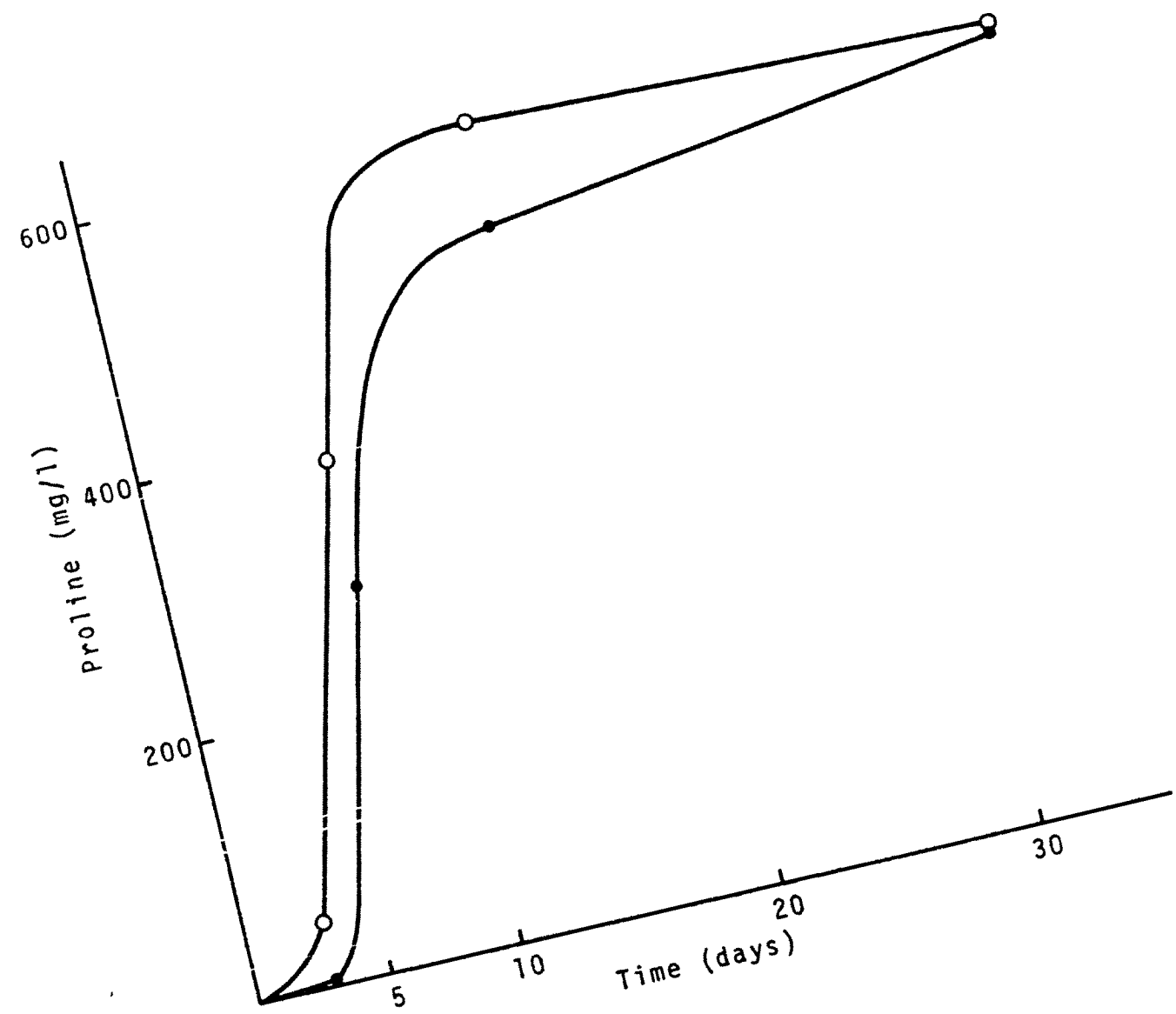

900
800
700
600
500
400
300
200
100
Proline (mg/l)
10
20
30
Time (days)
8
7
6
pH
9
8
7
Log10 number/ml

about 6.2, the greatest changes occurring in the control medium with yeast extract, and less markedly in the medium without yeast extract. Proline was not measured before the 3rd day, but as seen in other experiments, medium proline content decreases during the first day. After the cells have reached their stationary growth phase, a decrease in numbers takes place, most rapidly for cells in the medium containing Trypticase without yeast extract and slowest in SLB. The decrease in numbers was followed by an increase in pH, probably most rapidly in SLB (pH 7.5 after 35 days) and slowest in the medium containing only Trypticase (pH 7.2). The pH changes were probably caused by ammonia production from certain free amino acids by P. shermanii, P-59 (1). The ammonia content in Swiss cheese was much higher than in Italian cheeses, which indicates this proclivity of propionibacteria. The proline content increased most rapidly from the 3rd to the 15th day which correlates with the most rapid increase in pH. Proline production from yeast extract alone reached its maximum value after only 7 days and was thereafter constant. In SLB rapid proline production started between the 2nd and 3rd day, and a day later in the medium with Trypticase.

To better correlate the influence of yeast extract on proline production from Trypticase, the free proline content produced by P. shermanii, P-59, was corrected for free pro-

line in the initial medium, and for the proline produced from yeast extract (Figure 7). Exchange of yeast extract with a vitamin solution led to a later start on proline production, probably related to slower growth. Otherwise, proline production rates were quite similar up to the 10th day, and then slowed down faster in the medium with only Trypticase. Maximum amounts of proline were found after 15 days in SLB after which the total amount remained relatively constant. The proline content recovered from the medium with Trypticase alone continued to increase slowly up to 35 days. The amount of proline produced from Trypticase was, in both cases, the same after 35 days. These results indicate that yeast extract had no influence on the final amount of proline produced. Yeast extract, however, increased the growth rate and, thereby, the rate of proline production. Some unknown activating factor also may be involved, because the proline production continued slowly after 15 days in the Trypticase-containing medium. Considering the possible mechanisms for proline production, the results of these growth studies suggest that bacterial peptidases may be mainly involved. Biosynthesis may not be too important because the bulk of the proline production takes place when total cell numbers are decreasing. Some arginine breakdown also may be involved.

Figure 7. Proline production from Trypticase by P. shermanii, P-59, in SLB with and without yeast extract. The results are corrected for proline produced from yeast extract. Symbols: o , SLB, ● SLB in which yeast extract has been exchanged with a defined vitamin solution


600
400
200
Proline (mg/l)
5
10
20
30
Time (days)

Proline production from selected nitrogen sources

Trypticase, an enzymatic hydrolysate of casein, proved to be an excellent source for proline production by P. shermanii, P-59. Other nitrogen sources may be just as good, however, so it was decided to determine the influence of nitrogen sources from different protein sources. Enzymatic hydrolysates from casein, meat, and plant proteins were analyzed for total proline, total free proline, and peptide-bound proline (Table 16). Casitone, Trypticase, and Tryptone

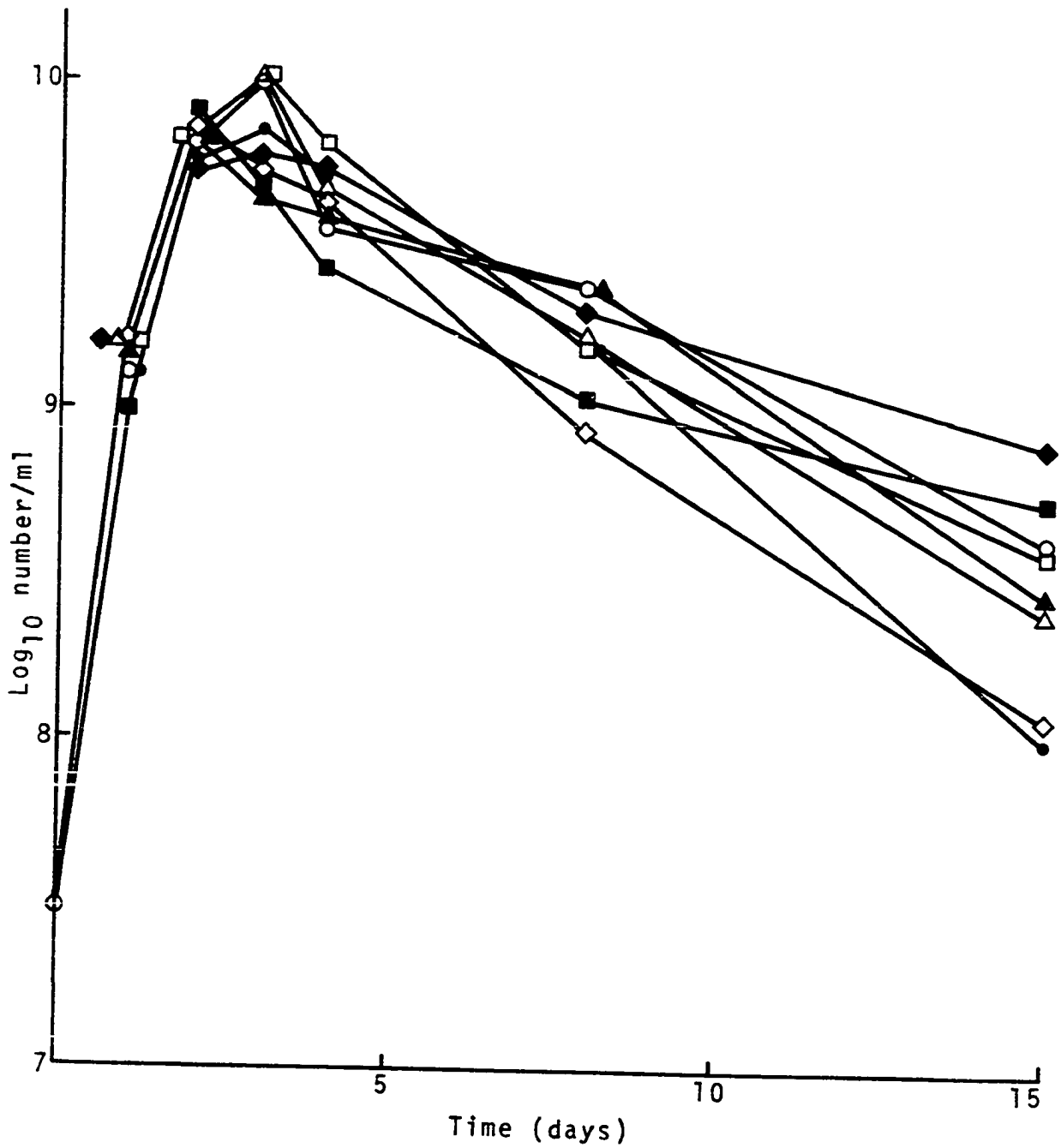
Table 16. Total and free proline present in different nitrogen sources

Nitrogen Source	Total proline (%)	Total free proline (%)	Amount of peptide-bond proline	Proline produced from N-source
Casitone	8.96	18.67	691.4	632.2
Trypticase	10.39	13.76	868.5	685.2
Tryptone	9.33	23.39	668.0	593.7
Peptone	12.01	21.13	904.9	228.0
Proteose-Peptone	7.20	12.48	605.3	238.8
Proteose-peptone no. 3	5.45	13.31	445.8	167.4
Phytone-peptone	2.79	20.06	182.9	170.6
Protone	3.67	8.47	318.9	240.4
Yeast extract	2.00	56.00	88.0	92.3

are all pancreatic digests of casein (21). The differences in total proline as measured were probably due to purity of the raw materials or water content of the test material. The proline content of Trypticase was very close to the value found in casein (60). The results indicated, of the tested materials, Tryptone was the most hydrolyzed and that Trypticase was least hydrolyzed. The meat hydrolysates included were Peptone, Proteose-peptone, and Proteose-peptone No. 3. Peptone contained the highest amount of proline of all of the enzymatic hydrolysates, and the proteose-peptone contained substantially less proline. This difference in proline content could be related to differences in raw material, and that peptone probably contained more connective tissues. The amounts of peptides found in Peptone and Tryptone are about the same (21, 40), but the peptide content was higher in the proteose-peptones (40), and they also contained higher amounts of larger peptides. Phytone-peptone is an enzymatic plant protein hydrolysate from soy meal (8) which contained low amounts of proline. Protone is an enzymatic hydrolysate of unknown source (21), containing a high amount of larger peptides (40).

The different nitrogen sources were exchanged with Trypticase in SLB, and growth and proline production by P. shermanii, P-59, were determined. Growth influences are shown in Figure 8. No large differences in maximum cell

Figure 8. Growth of P. shermanii, P-59, at 32 C, in media containing enzymatic hydrolysates of casein, meat proteins, and plant proteins. Symbols: ◇ , Control, SLB without Trypticase, o , 1% Trypticase, △ , 1% Tryptone, □ , 1% Casitone, ● , 1% Peptone, ▲ , 1% Proteose-peptone, ■ , 1% Proteose-peptone, No. 3, ◆ , 1% Phytone-peptone


10
9
8
7
Log10 number/ml
5
10
15
Time (days)

numbers were found with only slightly higher numbers present in media containing casein hydrolysates. Maximum cell numbers in casein hydrolysates were found after 3 days compared to 2 days for the other nitrogen sources but the numbers after 2 days were about the same for all nitrogen sources. Bacterial numbers decreased fastest with yeast extract and peptone as nitrogen sources, and slowest with Phytone-peptone. Except for Phytone-peptone, it seemed that cell survival increased with increasing amounts of peptides present.

Proline production in the different media is shown in Figures 9 and 10. Figure 9 reflects proline production during the first 4 days. No measurement was made on the first day but later experiments demonstrated that proline was utilized rather than produced during this time period. Otherwise, the results indicated that proline is produced fastest from the casein hydrolysates during the early stages, followed by the meat hydrolysates of which Peptone was the slowest. Induction of proline production was slowest for Phytone-peptone. Figure 10 shows proline production from the 4th to the 75th day. The most rapid proline production took place in the presence of casein hydrolysates. The fastest rate of proline production occurred with Trypticase, whereas Casitone was the slowest medium in this regard. Both Trypticase and Tryptone yielded their maximum proline content

Figure 9. Proline production by P. shermanii, P-59, at 32 C, during the early growth stages in media containing enzymatic hydrolysates of casein, meat proteins, and plant proteins. Symbols: ◇, Control medium, SLB without Trypticase, o , 1% Trypticase, Δ , 1% Tryptone, □ , 1% Casitone, ●, 1% Peptone, ▲ , 1% Proteose-peptone, ■ , 1% Proteose-peptone, No. 3, ◆ , 1% Phytone-peptone

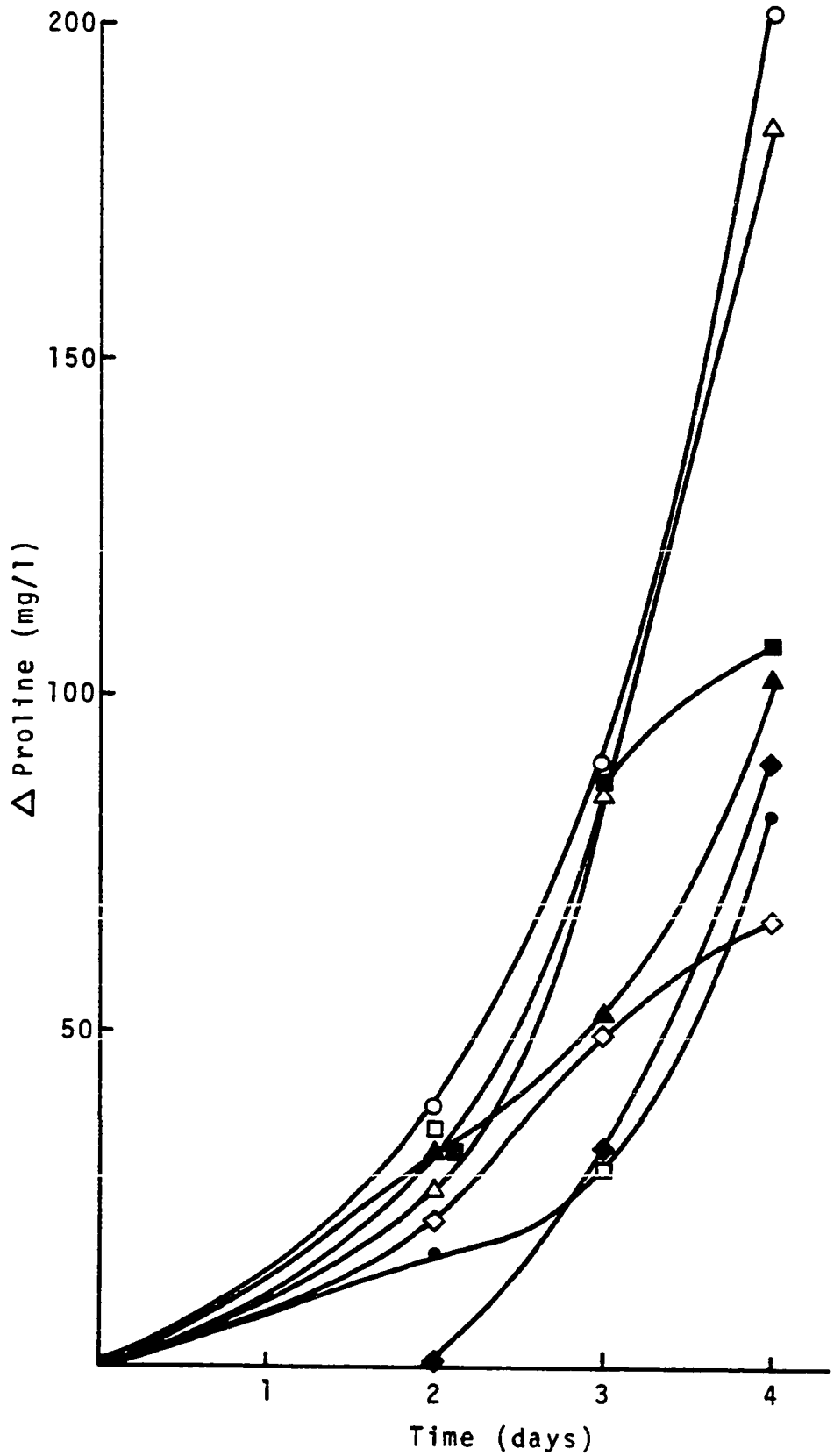
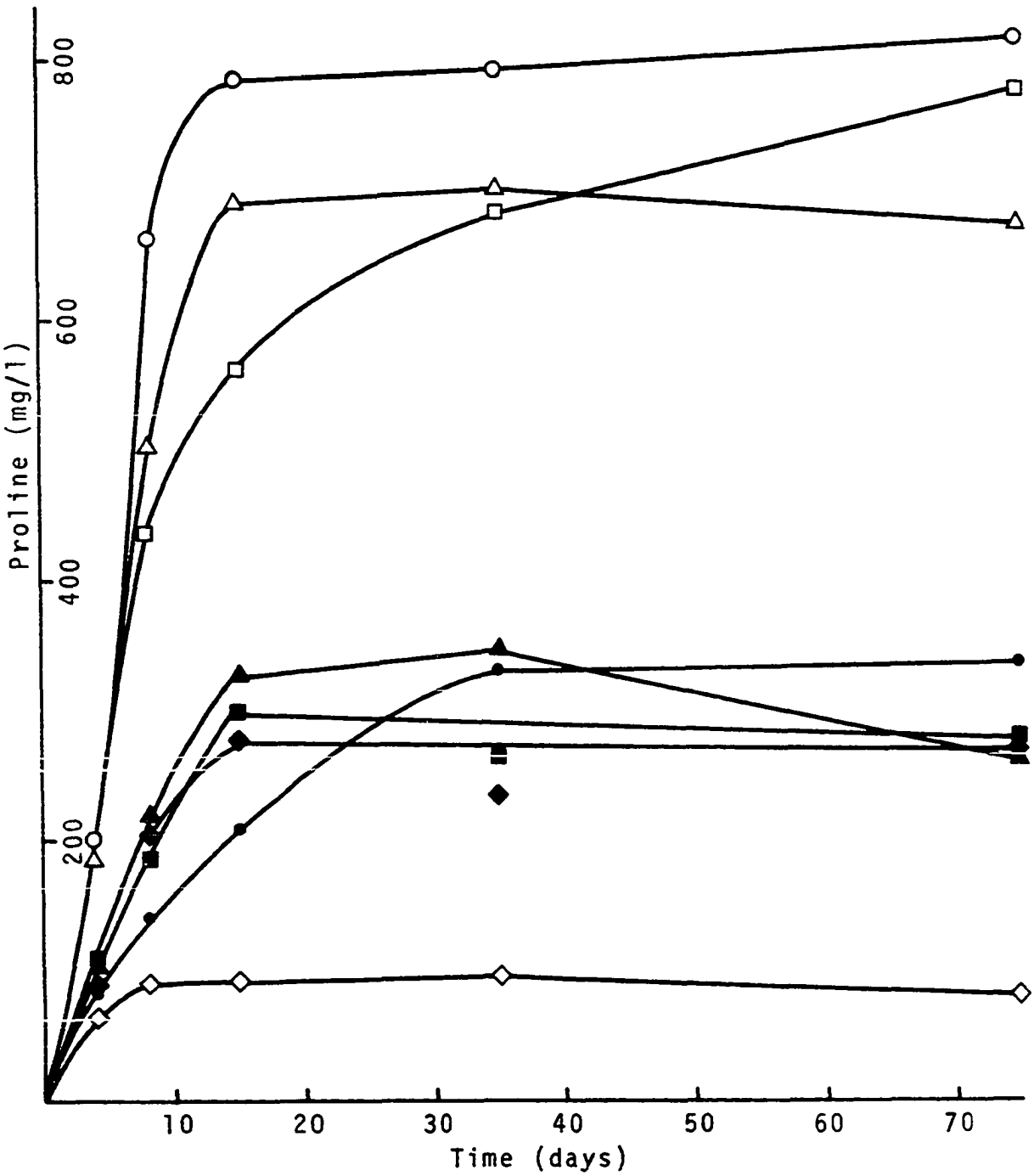

200
150
100
50
Δ Proline (mg/l)
1
2
3
4
Time (days)

Figure 10. Proline production by P. shermanii, P-59, at 32 C in media containing enzymatic hydrolysates of casein, meat proteins, and plant proteins. Symbols: ◇ , Control medium, SLB without Trypticase, o , 1% Trypticase, △ , 1% Tryptone, □ , 1% Casitone, ● , 1% Peptone, ▲ , 1% Proteose-peptone, ■ , 1% Proteose-peptone, No. 3, ◆ , 1% Phytone-peptone


Proline (mg/l)
800
600
400
200
10
20
30
40
50
60
70
Time (days)

after 15 days, while Casitone showed a continuous increase over the entire time period. The highest amount of proline was produced from Trypticase, which also contained, initially, the highest amount of peptides. Trypticase, however, was also the substrate which, after 75 days, still contained the highest amount of peptide-bound proline. The specificity of the peptidases will vary with size of peptides. The slower increase in proline production from Casitone may be caused by differences in peptide composition brought on by different degrees of hydrolysis, which, because of the specificity of the peptidases involved, will act slower on Casitone than on Tryptone. Enzyme specificity also may be the reason why Trypticase after 75 days contained the highest amount of proline-containing peptides. The amounts of proline incorporated in the cells of P. shermanii, P-59, were much smaller than the reamining peptide-bound proline. If biosynthesis from glutamic acid had been involved along with peptidase activity, one would have expected earlier and greater proline production because casein contains about 22% glutamic acid (60). If peptidase activities were not involved, the amount of proline produced was too high to have originated from glutamic acid. Even if arginine breakdown was involved, the proline produced would be too high because the arginine content of casein is only about 4%.

The proline production rate is much slower from meat and

plant hydrolysates, and is especially slow from Peptone. Much lower amounts of proline are produced. The difference between peptide-bound proline and free proline produced was much larger from the meat hydrolysates than from casein hydrolysates, which therefore are better substrates for proline production. The meat hydrolysates are probably obtained primarily from muscles, and it is known that the glutamic acid content is very high in meat proteins (107) compared to their proline content. Enzyme specificities were probably also involved in the slower proline production from Peptone. The results also indicated that media containing larger peptides did not slow down proline production. With Phytone-peptone and yeast extract, the total amount of proline produced equaled the peptide-bound proline. Glutamic acid content is three times higher than the proline content in yeast extract (21), and the glutamic acid content in soybean meal is 4.5 times higher (186). If an effective conversion was taking place, higher amounts of proline would have been present. However, small amounts may be produced from either glutamic acid or arginine, but, so far, peptidase activity seemed to be of primary importance in increasing the proline content of the media.

Influence of different concentrations of Trypticase on proline production

Peptides are known to have excellent nutritional value, but, in addition, some studies have indicated that certain nondegradable peptides, will accumulate and inhibit growth (226). Consequently, an experiment was designed to determine if an increase in amounts of Trypticase (peptides) would influence proline production from this nitrogen source. Reduction in cell numbers was not observed in media containing up to 4% Trypticase, but at a 5% level, slight inhibition of growth was observed. pH values seemed to be slightly more basic at the Trypticase lower concentrations (Table 17).

The proline production rate increased with Trypticase concentration (Figure 11), but after 35 days the rapid rate slowed or stopped. This cessation of activity may have been caused by enzyme inactivation at 32 C, the temperature of incubation. The proline concentrations produced by variation in Trypticase concentrations, were related to a 1% nitrogen basis to show the production (Figure 12). After 15 days, similar amounts were produced from 0.1 to 1.0% Trypticase; at higher concentrations, the free proline content decreased indicating substrate saturation (124). By increasing the time of incubation, the results showed that after 75 days, the amount of proline seemed to be constant per 1% Trypticase up to a 3% medium concentration. The lower amounts of proline

Table 17. Maximum cell numbers and pH changes by Propionibacterium shermanii, P-59, during growth in Sodium lactate broth with different concentrations of Trypticase

Concentration of Trypticase in %	Maximum cell numbers/ml x 10^5	Time (days) 3	15	35
0	49,000	6.45	6.95	7.60
0.1	48,000	6.40	7.18	7.40
0.5	45,000	6.30	7.20	7.30
1.0	56,000	6.58	7.20	7.35
2.0	50,000	6.48	7.15	7.52
3.0	56,000	6.50	7.10	7.30
4.0	50,000	6.50	7.10	7.20
5.0	42,000	6.58	7.15	7.20

Figure 11. Influence of different concentrations of Trypticase on proline production by P. shermanii, P-59, in SLB. Symbols: ◇, Control, SLB without Trypticase, o, 0.1% Trypticase, △, 0.5% Trypticase, □, 1% Trypticase, ◆, 2% Trypticase, ●, 3% Trypticase, ▲, 4% Trypticase, ■, 5% Trypticase

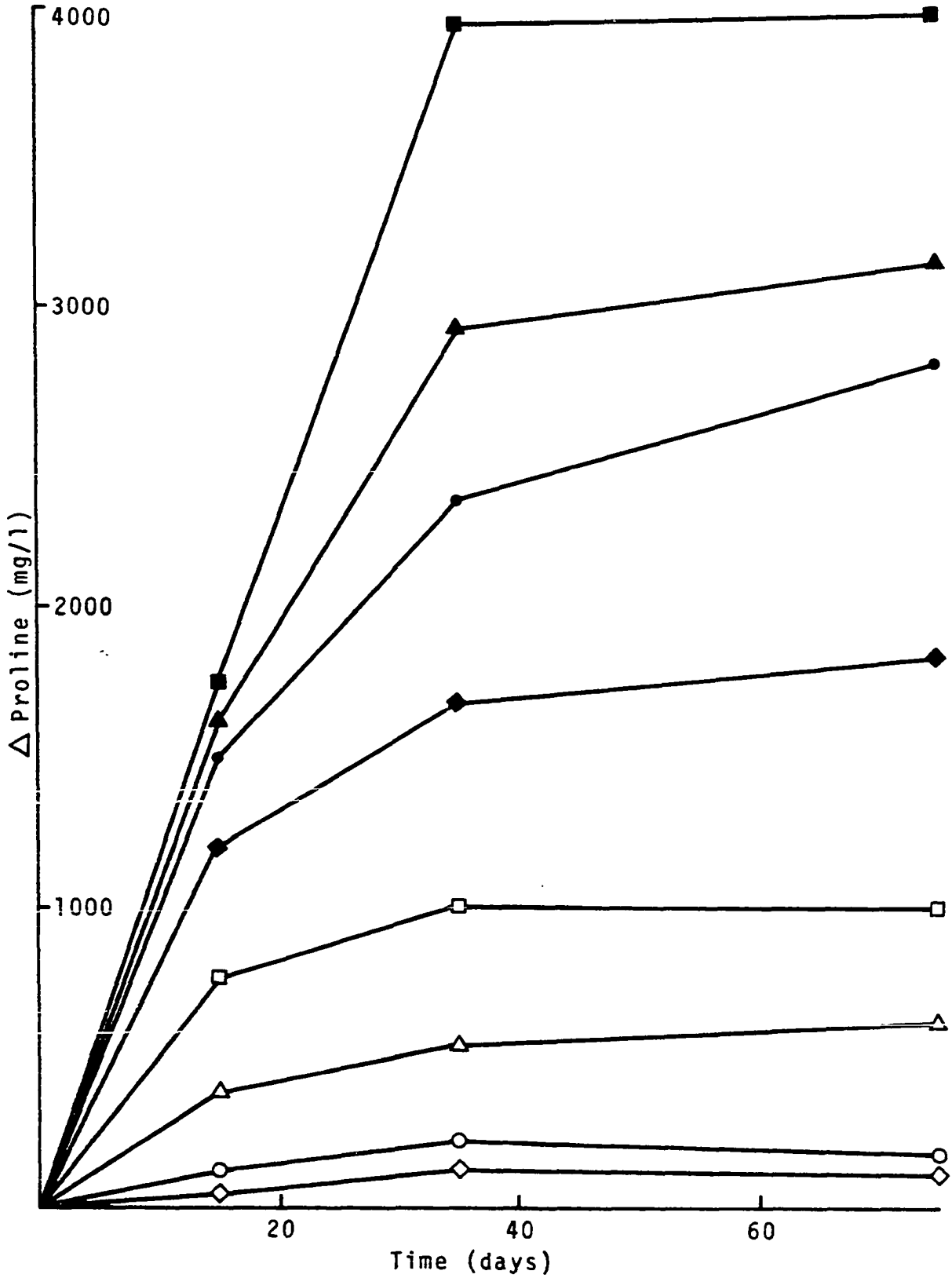
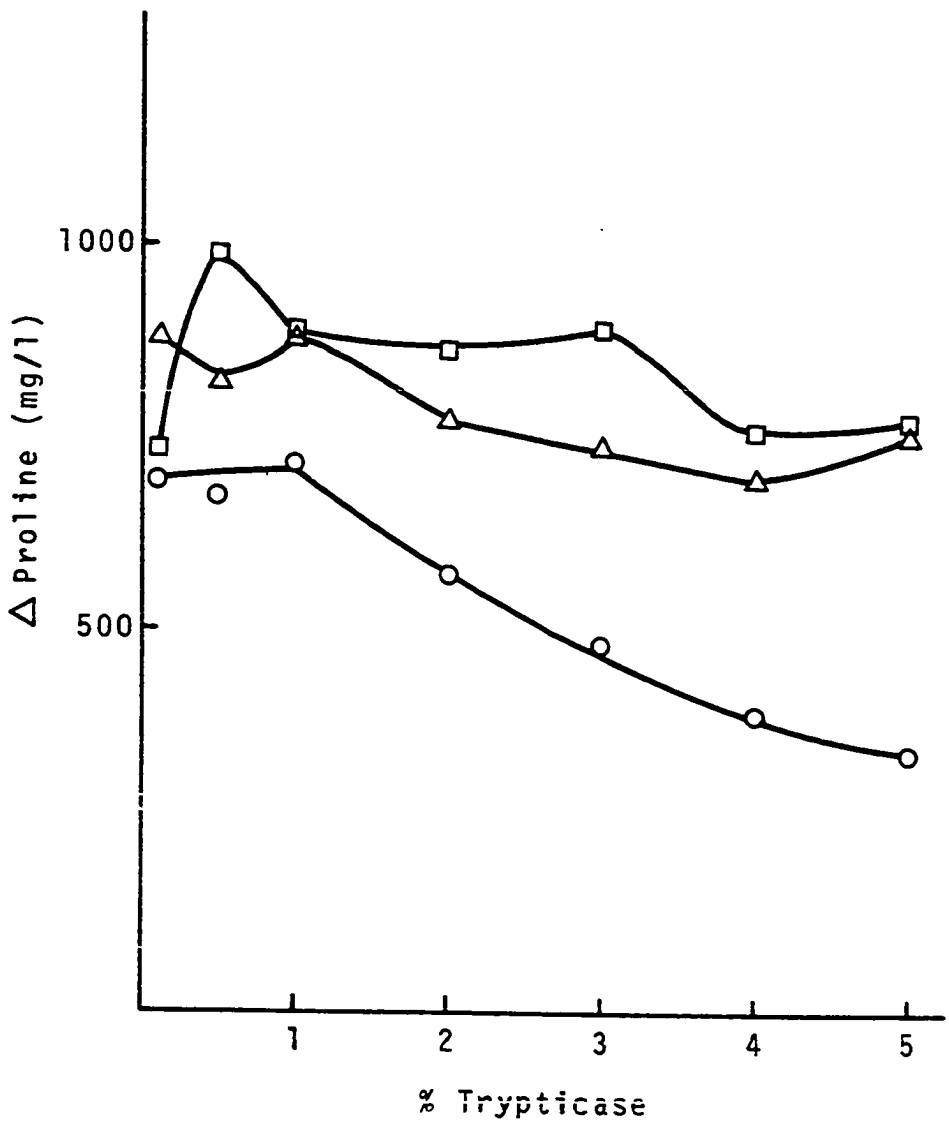

4000
3000
2000
1000
Δ Proline (mg/l)
20
40
60
Time (days)

Figure 12. Proline produced by P. shermanii, P-59, from media containing different amounts of Trypticase calculated to show proline production per 1% Trypticase in all cases. Symbols: o , 15 days, Δ , 35 days, □ , 75 days


1000
500
Δ Proline (mg/l)
1
2
3
4
5
% Trypticase

produced at 4 and 5% Trypticase were probably due to enzyme inactivation. Inconsistencies at 0.1 and 0.5% may be due to analytical error.

Influence of Protone and Peptone addition on proline production from Trypticase

Of the various nitrogen sources, Trypticase was found to be the best for proline production. Trypticase contains a large number of smaller peptides. The question, therefore, could be asked: would large peptides influence proline production from Trypticase? Protone, an enzymatic hydrolysate from an unknown protein source, contains large amounts of large peptides (21, 40). Protone contains 3.7% proline, of which only 8.5% is free.

The maximum cell numbers of P. shermanii, P-59, in media containing mixtures of Trypticase and Protone or Protone alone, were found after 2 days, in contrast to 3 days with Trypticase alone, as shown in Figure 13. Growth was slowest when Protone was the sole nitrogen source. The survival rate after 15 days was not very different, but cell numbers were somewhat higher in Protone and in the media containing 0.1 and 0.5% Protone, indicating that media containing the larger peptides somehow stabilized the cells. Proline production during the first 4 days showed that Protone was a poor source of proline (Figure 14). The fastest proline production rate occurred with Trypticase as a nitrogen source. By adding

Figure 13. Growth of P. shermanii, P-59, at 32 C, in media containing Protone or Trypticase, or mixtures of Trypticase with Protone or Casein. Symbols: ◇, Control medium, SLB without Trypticase, ○, 1% Trypticase, △, 1% Protone, □, 1% Trypticase + 0.1% Protone, ●, 1% Trypticase + 0.5% Protone, ▲, 1% Trypticase + 0.1% Casein, ■, 1% Trypticase + 0.5% Casein

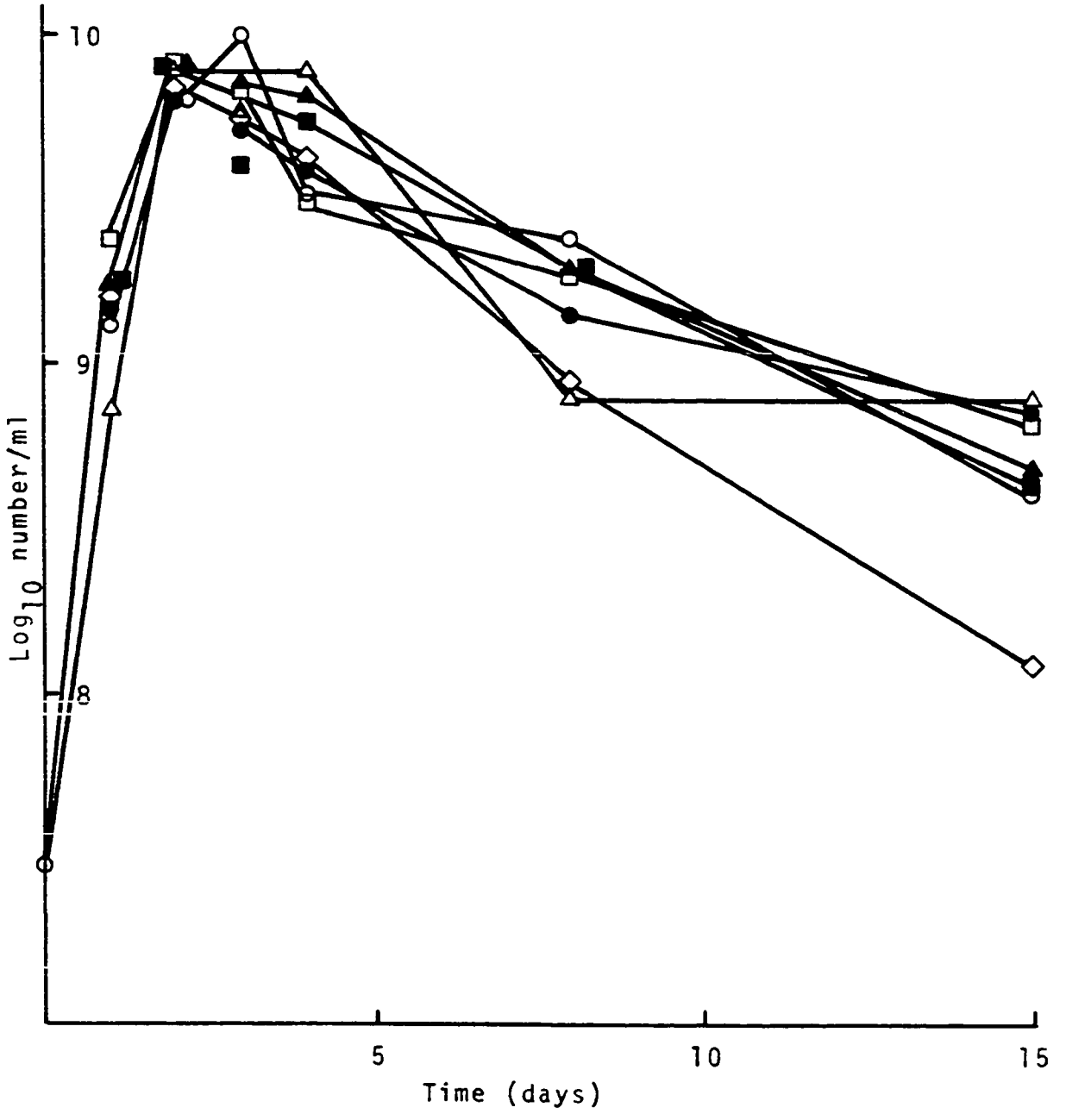
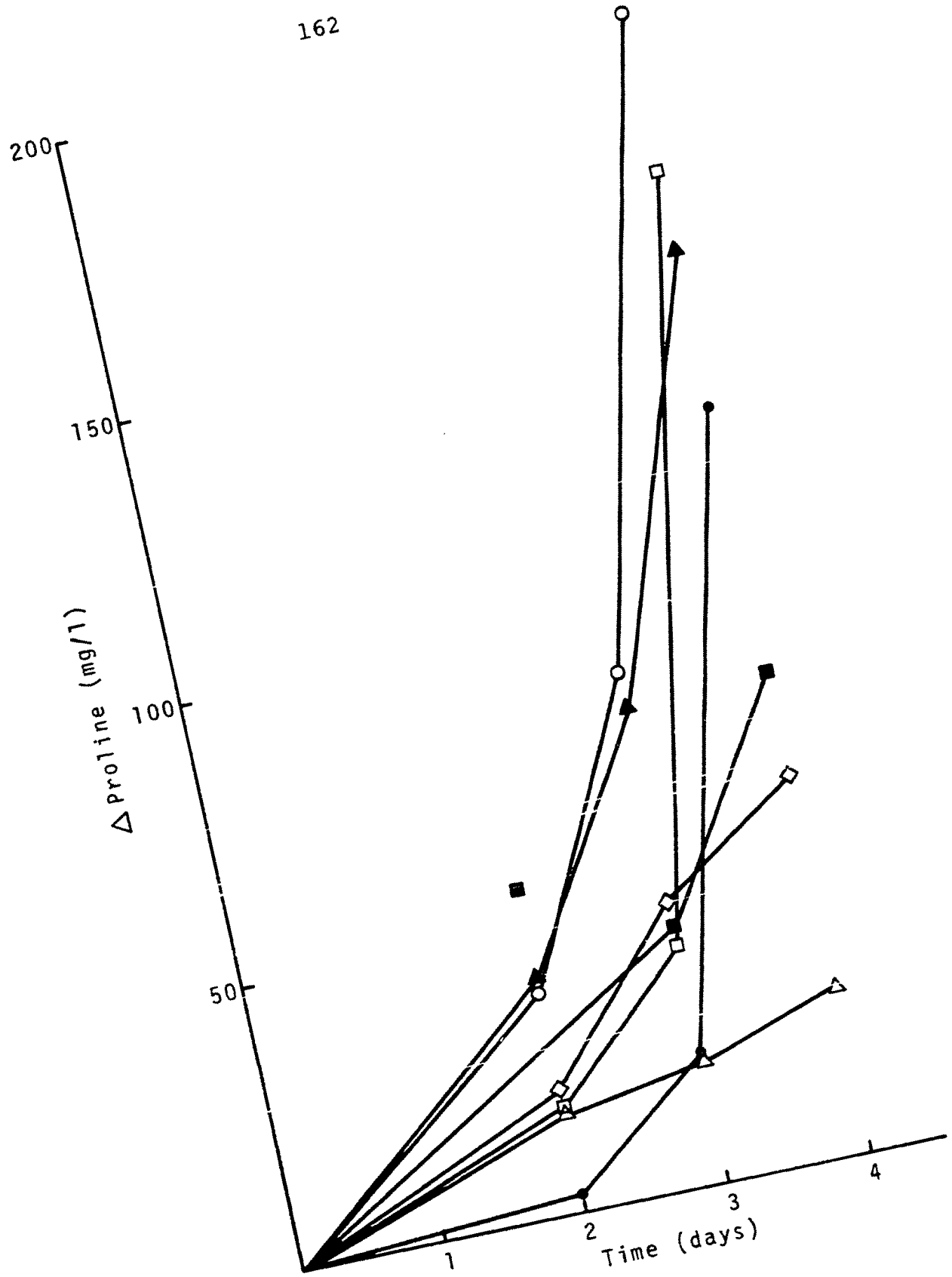

10
9
8
Log10 number/ml
5
10
15
Time (days)

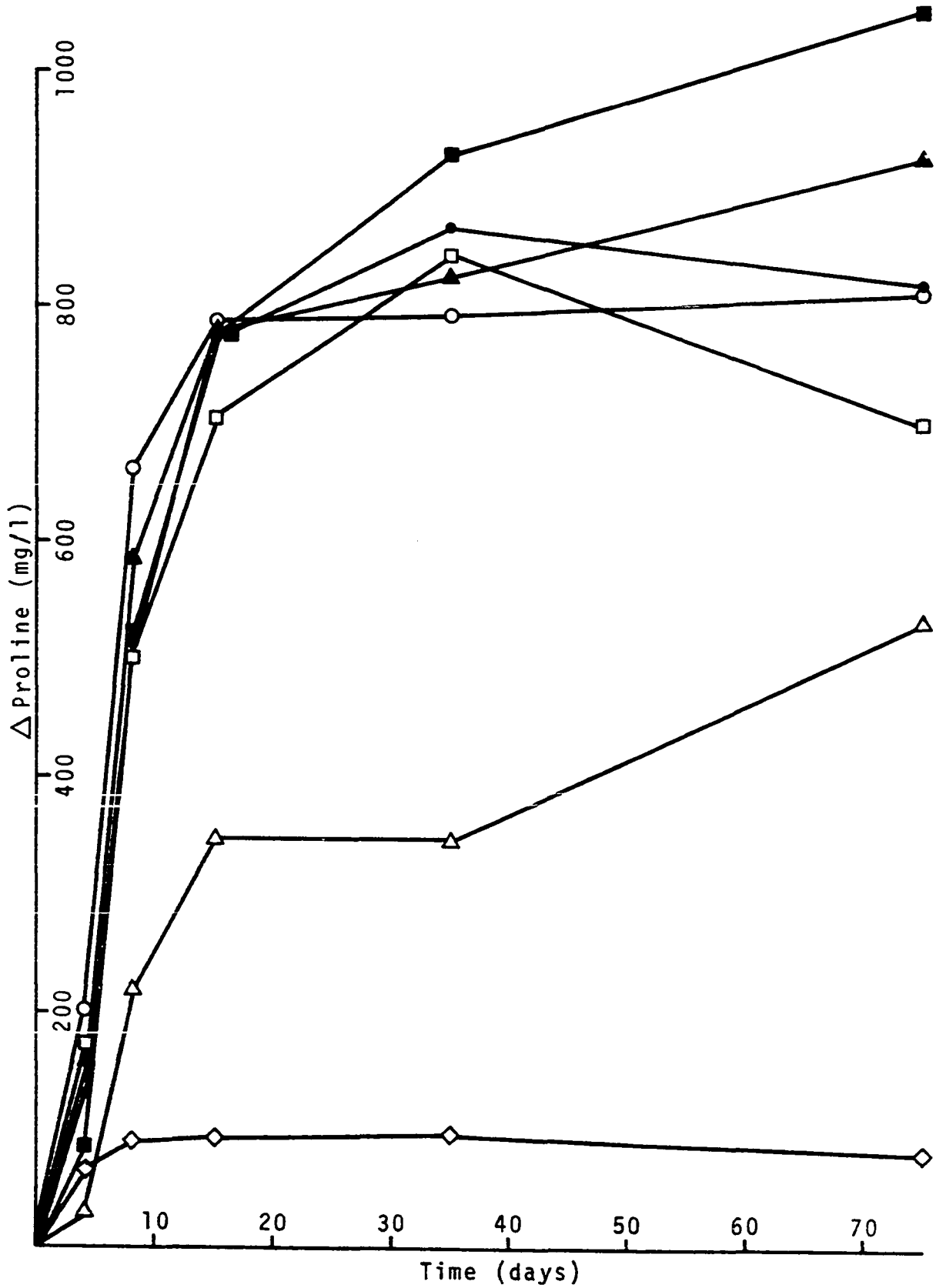
Figure 14. Proline production by P. shermanii, P-59, at 32 C, during the early growth stages in media containing Protone or Trypticase, or mixtures of Trypticase with Protone or Casein. Symbols: ◇ , Control medium, SLB without Trypticase, o , 1% Trypticase, △ , 1% Protone, □, 1% Trypticase + 0.1% Protone, ● , 1% Trypticase + 0.5% Protone, ▲ , 1% Trypticase + 0.1% Casein, ■ , 1% Trypticase + 0.5% Casein


200
150
100
50
Δ Proline (mg/l)
1
2
3
4
Time (days)

0.1% Protone, the rate of proline production decreased, and addition of 0.5% Protone also led to a slower initiation of proline production. Figure 15 shows that the addition of Protone to Trypticase definitely led to a slower production rate with smaller free proline contents after 7 days. After 15 days, the amounts of proline were the same in the medium containing Trypticase alone or with 0.5% Protone, and were lower in the medium containing 0.1% Protone. Larger amounts of proline should have been produced in the media containing 0.1 and 0.5% Protone in addition to Trypticase, because of larger contents of peptide-bound proline. After 35 days, however, the proline content produced from Trypticase was about the same in the control medium and in the medium with 0.1% Protone. Less proline than expected was produced from the medium containing 0.5% Protone.

These results indicated that larger peptides slow down proline production, and that less proline than would be expected is produced. Growth differences were very small, and would probably have little, if any, influence on the rate of proline production. Differences in autolysis, which will be discussed in greater detail later, may influence the rate, but not the final amount of proline. The most probable causes for these differences are possibly that the larger peptides may have some inhibitory effect on the peptidases involved (124), or that enzyme activity towards larger

Figure 15. Proline production by P. shermanii, P-59, at 32 C, in media containing Protone or Trypticase, or mixtures of Trypticase with Protone or Casein. Symbols: ◇ , Control medium, SLB without Trypticase, ○ , 1% Trypticase, △ , 1% Protone, □, 1% Trypticase + 0.1% Protone, ● , 1% Trypticase + 0.5% Protone, ▲ , 1% Trypticase + 0.1% Casein, ■ , 1% Trypticase + 0.5% Casein


1000
800
600
400
200
Δ Proline (mg/l)
10
20
30
40
50
60
70
Time (days)

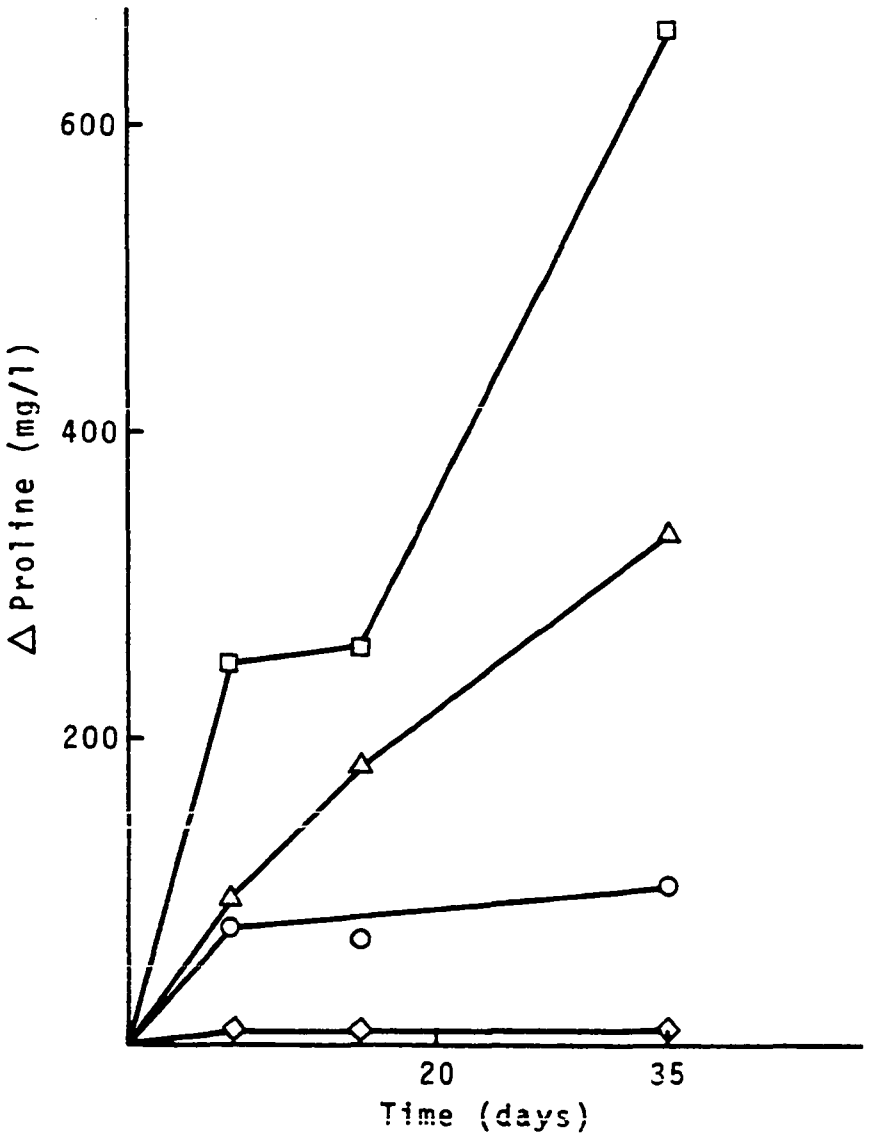
peptides may be lessened. Another possibility is, of course, that the enzymes are more easily destabilized, when greater amounts of large peptides are present. This thought is in opposition to the idea that larger molecules very often stabilize enzymes. The decrease in proline content after 35 days was most likely caused by degradation, because the experiments were performed in a dynamic system, where both production and degradation take place simultaneously. It is known that propionibacteria can degrade amino acids (1).

In an earlier experiment it was observed that the rate of proline production from Peptone was much slower than from Trypticase, and that the amount of peptide-bound proline in the spent medium was very high after proline production had ceased. This result may indicate that Peptone could have some deleterious effect on proline production from Trypticase. Both nitrogen sources contained large amounts of both peptide-bound proline and small peptides. The maximum cell numbers and pH changes during growth of P. shermanii, P-59, are shown in Table 18. Significant differences in maximum cell numbers were not found, but it was observed that pH changes were smaller in media containing Peptone. Figure 16 shows proline production in media containing Peptone as the sole nitrogen source. Proline production by P. shermanii, P-59, seems to continue over the entire 35-day experimental period with Peptone concentrations

Table 18. Maximum cell numbers and pH changes by Propionibacterium shermanii, P-59, in media containing mixtures of Trypticase (BBL) and Peptone (Difco)

Concentration of T and P	Maximum cell numbers/ml x 10^5	Time (days) 3	7	15
Control	49,000	6.40	6.70	6.98
0.1% T	55,000	6.38	6.60	7.45
0.5% T	50,000	6.30	6.70	7.40
1.0% T	48,000	6.39	6.73	7.25
3.0% T	54,000	6.50	6.73	7.15
0.1% T + 1.0% P	59,000	6.78	6.58	7.15
0.5% T + 1.0% P	56,000	6.32	6.50	7.70
1.0% T + 0.1% P	52,000	6.32	6.60	7.32
1.0% T + 0.5% P	54,000	6.35	6.48	6.95
1.0% T + 1.0% P	52,000	6.30	6.30	6.75
1.0% T + 3.0% P	66,000	6.28	6.47	6.50
3.0% T + 1.0% P	42,000	6.30	6.32	7.10
0.1% P	49,000	6.30	6.50	6.85
0.5% P	52,000	6.28	6.43	6.75
1.0% P	64,000	6.25	6.12	6.80
3.0% P	51,000	6.15	6.25	6.60

Figure 16. Influence of different concentrations of Peptone on proline production by P. shermanii, P-59, in SLB containing Peptone instead of Trypticase. The results are corrected for amounts of proline produced from yeast extract. Symbols: ◇ , 0.1% Peptone, o , 0.5% Peptone, △ , 1% Peptone, □ , 3% Peptone

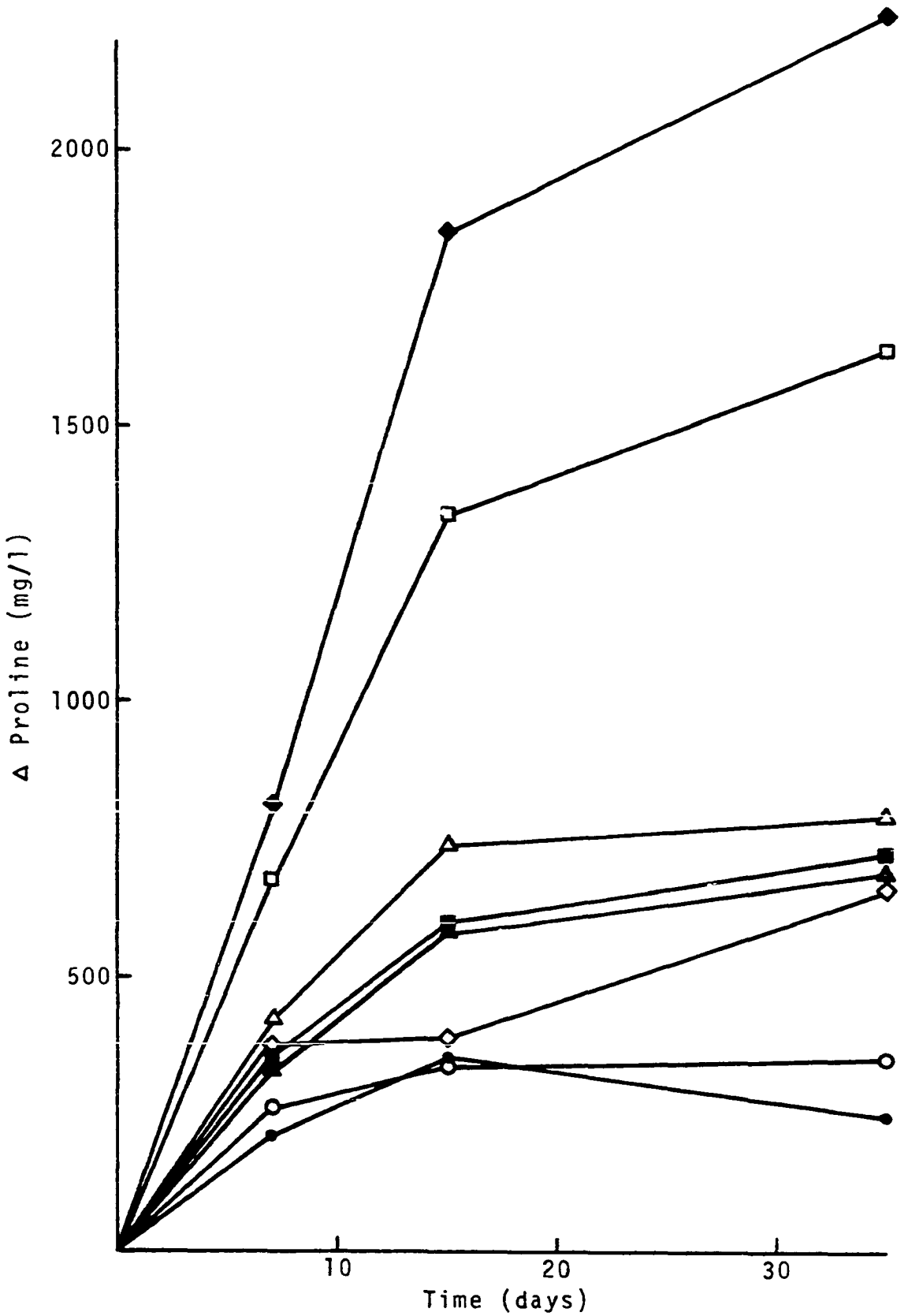

600
400
200
Δ Proline (mg/l)
20
35
Time (days)

of 1 and 3%. The experiment was stopped after 35 days, because an earlier experiment showed that only very small increases with a prolonged incubation period could be expected. The amount of proline produced from 3% Peptone was less than expected when compared with the amount produced from 1%.

The results from this experiment were used to correct the amount of proline produced by P. shermanii, P-59, in mixtures of Trypticase and Peptone. The proline production from media containing Trypticase corrected for amounts produced from Peptone is shown in Figure 17. Addition of 1% Peptone to a medium containing 0.1% Trypticase, had no effect on the proline production from Trypticase. However, addition of 1% Peptone to 0.5% Trypticase led to less proline production. In a mixture containing 1% Trypticase and 0.1% Peptone, no influence on proline production was observed, but with additions of 0.5, 1, and 3% Peptone, noticeable smaller amounts of proline were produced. In a mixture containing 3% Trypticase and 1% Peptone, the effect was even clearer. The differences in amounts of proline produced in the control and in the mixtures increased with increasing amounts of both Peptone and Trypticase. Peptone definitely had an inhibitory effect on proline production from Trypticase.

The results from this group of experiments indicate that proline peptidases must be important for proline production. Biosynthesis from glutamic acid or arginine degradation,

Figure 17. Influence of Peptone on proline production from Trypticase by P. shermanii, P-59, in SLB containing Trypticase alone or mixtures of Trypticase and Peptone. The proline contents are corrected for amounts of proline produced from yeast extract and Peptone. Symbols: o , 0.5% Trypticase, ● , 0.5% Trypticase + 1% Peptone, △ , 1% Trypticase, ▲ , 1% Trypticase + 0.5%% Peptone, ■ , 1% Trypticase + 1% Peptone, ◆, 3% Trypticase, □ , 3% Trypticase + 1% Peptone, ◇ , 1% Trypticase + 3% Peptone


2000
1500
1000
500
Δ Proline (mg/l)
10
20
30
Time (days)

however, can not be excluded, and proteinase activity also may be involved. Enzymatic hydrolysates from casein were the best sources for proline, while meat hydrolysates, and especially, Peptone, were poor sources even though they contained quite large amounts of proline. The amount of proline produced from Phytone-peptone, a soy meal hydrolysate, was equal to the peptide-bound proline. Proline production from meat hydrolysates was much slower than from casein hydrolysates and was especially slow from Peptone. Smaller amounts of proline were produced from Trypticase when Peptone was added to the medium. Peptone contained much more proline than would be expected from muscle proteins indicating that connective tissues, containing large amounts of proline, were present in the preparation of Peptone. Peptone would then also contain hydroxyproline peptides. Hydroxyproline is structurally close to proline, but it has been observed that some proline peptidases do not act on hydroxyproline peptides (195). The reason for the inhibitory effect of Peptone, may possibly be caused by competitive inhibition of some proline peptidases (124).

Proline production in a defined medium

Biosynthesis of proline from glutamic acid may provide another way by which proline may be produced in large amounts. Industrially, certain microorganisms have been used for proline

production, but they have all been mutants (87, 257). Baich and Pierson (7) isolated a proline-excreting mutant from E. coli in which the first enzyme in the biosynthetic pathway (Figure 1) (usually regulated by feed-back inhibition) is not inhibited by proline.

The first logical step would be to determine if P. shermanii, P-59, contains the necessary enzymes for proline biosynthesis. It was necessary to use a defined medium for this experiment. Kurmann (116) had previously developed a synthetic medium containing casein, which, unfortunately, makes the medium less defined. Therefore, it was decided to use a medium developed for growth of Staphylococcus aureus (Patte, private communication). Optimum growth was needed and the effect of vitamins other than those required (20) was examined. Results are shown in Table 19.

The data from this experiment were used to determine if proline could be produced from a defined medium. One medium containing proline and another without proline was used with SLB being used for comparison. Maximum cell numbers were reached after 4 days in the defined medium in comparison with 3 days in SLB. Although growth was slower no large difference in maximum numbers was found between the defined media and SLB. Table 20 shows the changes in proline content in the medium during this experiment. In the medium containing proline initially, a continuous decrease in proline

Table 19. Influence of vitamins on optimum cell yield in defined medium[a]

Strains	Vitamin Solutions							
	I	II	III	IV	V	VI	VII	VIII
P. freudenreichii, P-19	1	=	=	+	=	=	=	-
P. pentosaceum, P-9	1	=	=	+	+	+	+	=
P. shermanii, P-24	1	=	=	+	=	=	-	-
P. shermanii, P-59	1	=	=	=	+	+	=	-
P. zeae, P-35	1	=	=	+	=	+	+	-

[a]Medium containing only vitamin solution I = control medium. Growth yield is reported as 1 for control medium, increase in yield +, decrease in yield -, no effect =.

Table 20. Total free proline (mg/l) in defined medium compared with Sodium lactate broth during growth of Propionibacterium shermanii, P-59, at 32 C

Medium	Total free proline (mg/l)							
	0	2	3	4	8	15	35	75
defined medium + proline	108.4	106.8	91.2	90.0	90.6	85.5	82.2	-
Defined medium - proline	0	1.5	0.6	2.1	2.7	4.8	4.8	4.2
Sodium lactate broth	294.5	333.25	384.4	496.0	957.9	1081.9	1088.1	1109.0

was observed, occurring most rapid during the first 3 days. In the defined medium, some increase was observed but only 4.8 mg/l was present after 15 days. This amount is very close to the proline content of the amino acid pool of P. shermanii, P-59, which was measured in a later experiment. Propionibacteria, therefore, appear to have the necessary enzymatic apparatus to synthesize proline, but the pathway is regulated. The medium contained sufficient glutamic acid so that if biosynthesis is important, a decrease in proline would not be expected in the medium containing proline, and a larger amount than 4 mg/l would have been expected in the medium without proline. It may be mentioned, however, that glucose was used as an energy source in this experiment, and that the pH of the media had fallen to about pH 4.4 after 5 days. This low pH may have inhibited proline production, but, in reference to other studies (86, 258), the proline production would be expected to follow.

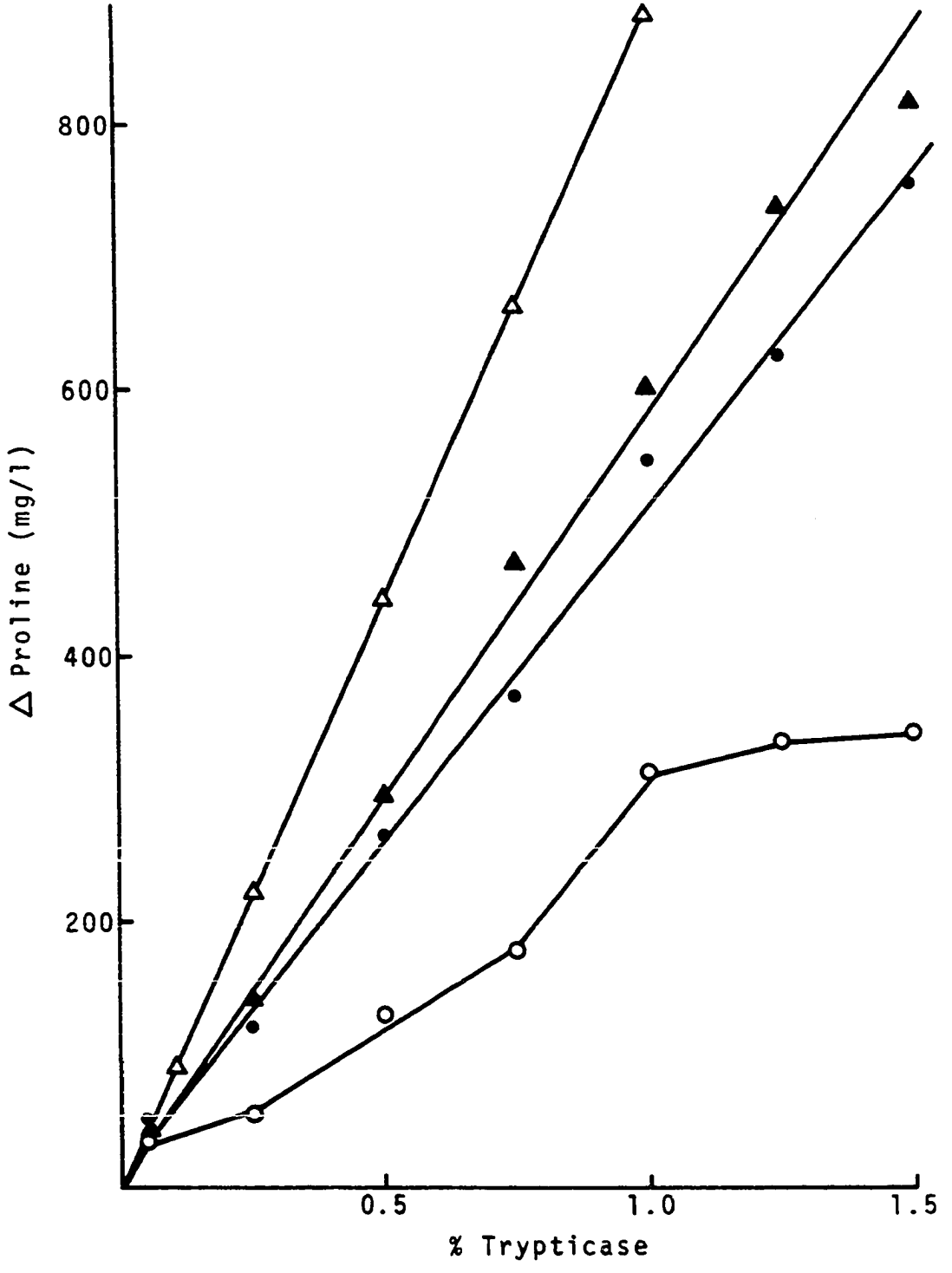
Proline production from media containing small amounts of Trypticase

If proline biosynthesis from glutamic acid is important for proline production by propionibacteria, larger amounts would be expected to be produced in media containing Trypticase. Trypticase is an enzymatic hydrolysate from casein containing 10.4% proline and about 22% glutamic acid (60). If both peptidase and biosynthesis were involved, one would

expect higher values of proline than the sum of free and peptide-bound proline because of the high glutamic acid content. If peptidases were not involved, however, lower values of proline would be expected to develop than were found in the earlier experiments.

To pursue this question, information was used from an earlier experiment in which proline production was measured as a function of Trypticase concentration. For this experiment, small Trypticase concentrations were used so that the amounts would not influence the growth. The results are shown in Figure 18. After 7 days, the production of proline as a function of Trypticase concentration was not linear, but after 15 and 35 days it was linear. The maximum amount of proline in the medium was reached after 35 days. The free proline content produced by P. shermanii, P-59, never reached the amount found totally in the medium. The difference between the proline produced by P. shermanii, P-59, and the total proline content of Trypticase increases linearly with concentration. For instance, at 0.5% Trypticase, the difference between total proline produced and total proline is 150 mg/l, at 1% it is 300 mg/l. This strongly indicates that proline biosynthesis is not important in the production of proline by propionibacteria.

Figure 18. Proline produced from Trypticase by P. shermanii, P-59, as a function of Trypticase concentration compared with total proline in an acid hydrolysate of Trypticase. Symbols: o, 7 days, ●, 15 days, ▲, 35 days, △, Total proline in Trypticase


800
600
400
200
Δ Proline (mg/l)
0.5
1.0
1.5
% Trypticase

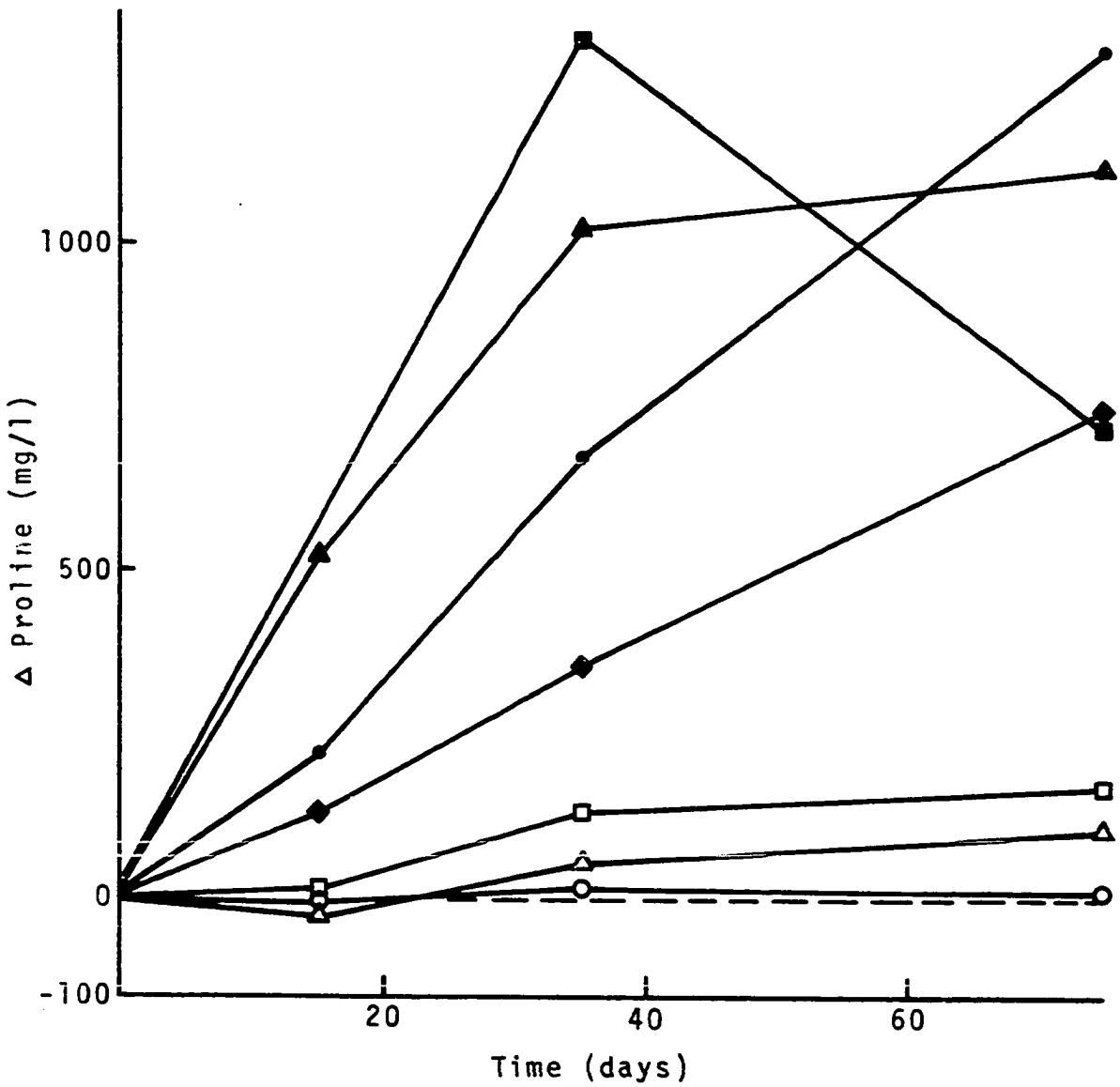
Proline production from Casamino acids

The earlier experiment strongly indicated that proline production by propionibacteria through biosynthetic pathways was of little importance. To further pursue proline production from amino acids, a medium containing different amounts of Casamino acids was used. The growth of P. shermanii, P-59, was inhibited at higher concentrations of Casamino acids (4 and 5%) as shown in Table 21. The pH changes also were much less than in SLB. Proline was produced from Casamino acids, but in much lower amounts than from Trypticase. The rate of proline production is shown in Figure 19. During the first 15 days, the proline content decreased slightly in media containing 0.1 and 0.5% Casamino acids, and increased at all other concentrations. At 35 and 75 days, the amount of proline had increased at all concentrations of Casamino acids. The rate of proline production was much slower than from Trypticase, and the amount of proline produced from 1% Casamino acids after 15 days was only 15 mg/l compared to 780 mg/l from 1% Trypticase. After 35 days, 130 mg/l was produced from Casamino acids compared to 795 mg/l from 1% Trypticase. This relationship fits for all other concentrations of Casamino acids. If proline biosynthesis from glutamic acid was important, as mentioned earlier, a production rate much more closely related to the growth curve (86, 258) would be expected.

Table 21. Maximum cell numbers and pH changes by Propionibacterium shermanii, P-59, during growth in a medium containing different concentrations of Casamino acids with defined amounts of vitamin

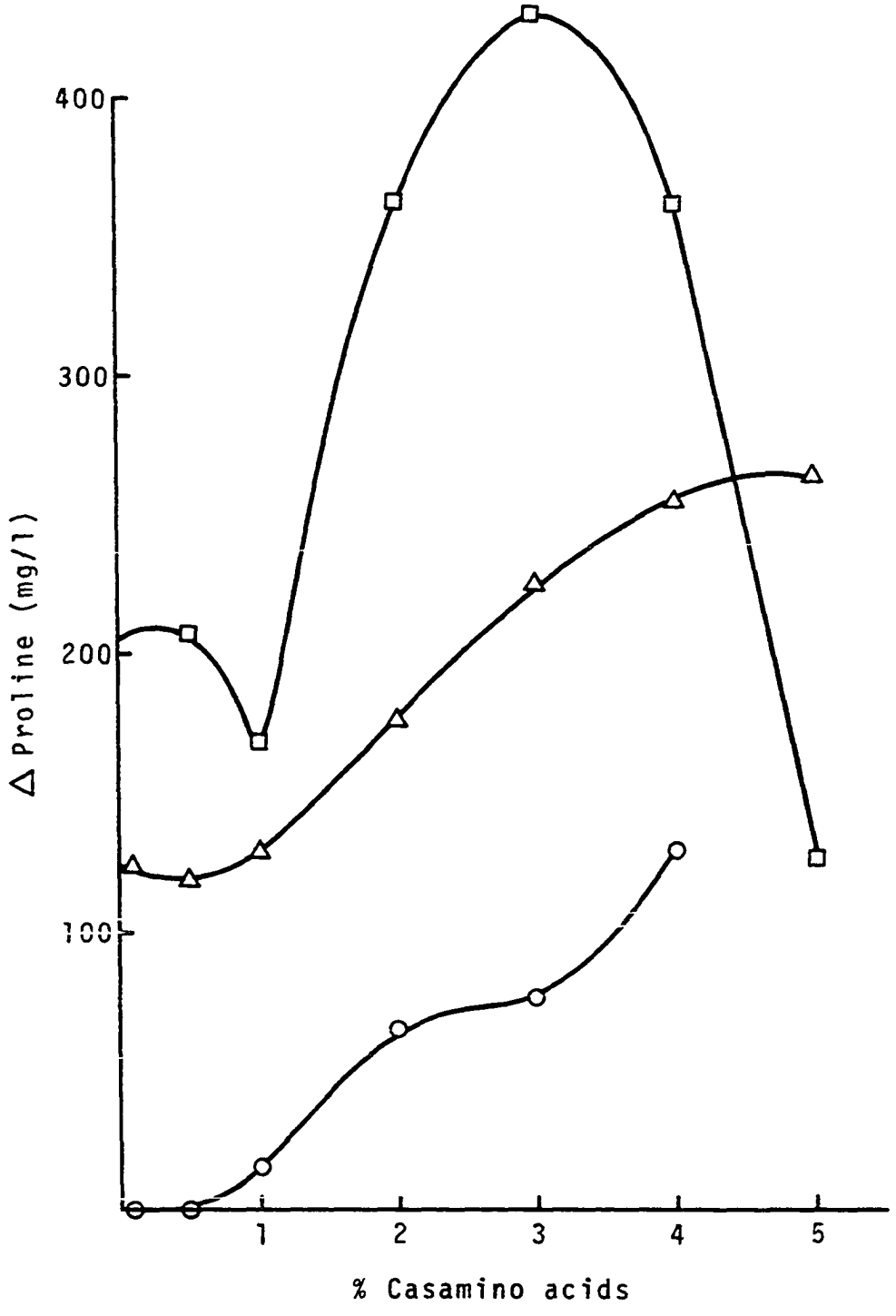
Concentration of Casamino acids in %	Maximum cell numbers/ml x 10^5	Time (days) 3 (pH)	15 (pH)	35 (pH)
0.1	57,000	6.42	6.70	6.80
0.5	45,000	6.22	6.50	6.92
1.0	50,000	6.23	6.82	6.80
2.0	62,000	6.20	6.70	6.75
3.0	44,000	6.16	6.45	6.60
4.0	31,000	6.12	6.55	6.55
5.0	12,000	6.08	6.50	6.12

Figure 19. Influence of different concentrations of Casamino acids on proline production by P. shermanii, P-59, in media in which yeast extract has been exchanged with vitamins. Symbols: o , 0.1% Casamino acids, Δ , 0.5% Casamino acids, □ , 1% Casamino acids, ◆ , 2% Casamino acids, ● , 3% Casamino acids, ▲ , 4% Casamino acids, ■ , 5% Casamino acids


1000
500
0
-100
Δ Proline (mg/l)
20
40
60
Time (days)

The concentration of proline as related to different Casamino acid concentrations, was calculated to show proline production per 1% Casamino acids (Figure 20). In contrast to the results obtained when Trypticase was incorporated in the medium, the results show that the amount of proline produced, increased with increasing amounts of Casamino acids except at the higher concentrations. At the greater levels, large fluctuations, probably related to growth variations occurred. Glutamic acid constitutes about 22% of Casamino acids (60), and the conversion to proline will only be from less than 1 to 8% of the total glutamic acid content in 1% Casamino acids. As mentioned earlier, earlier and faster proline production by the biosynthetic pathway (86, 258) would be expected. Another possibility might be proline production from arginine through ornithine which constitutes a degradative pathway. This possibility may correlate with the low arginine content observed in regular Swiss cheeses, but a note of care is necessary because the arginine content also was low in Italian cheeses. Proline production from arginine, with ornithine as an intermediate, has been observed in many microorganisms (30, 144, 218, 244). Because this is a degradative pathway not regulated by feed-back inhibition, this reaction should be active over a long time period. The arginine content of Casamino acids is only 4% (60) which would lead to a more efficient conversion than from glutamic

Figure 20. Proline production by P. shermanii, P-59, from media containing different concentrations of Casamino acids calculated to show proline production per 1% Casamino acids in all cases. Symbols: o , 15 days, Δ , 35 days, □ , 75 days


400
300
200
100
Δ Proline (mg/l)
1
2
3
4
5
% Casamino acids

acid (from 4 to 40% at 1% Casamino acids, and from 15 to 90% for 2% Casamino acids). This leads to the assumption that the proline produced from Casamino acids is mainly from the arginine present. This thought also would explain the continued slow increase in proline content from Trypticase after 15-days incubation.

Effect of glutamic acid on proline production

Earlier experiments indicated that glutamic acid was not a precursor for proline production. To further substantiate these results, an experiment was performed in which different amounts of glutamic acid were added to a control medium containing 0.5% Casamino acids. Addition of glutamic acid in amounts from 0.5 to 8 g/l had no effect on the growth of propionibacteria. The results from after 35-days incubation with P. shermanii, P-59, are shown in Table 22.

Differences in amounts of proline produced were not observed with increasing glutamic acid concentrations. The biosynthetic pathway from glutamic acid has been shown to be regulated by feed-back inhibition by proline (7). The free proline content in the medium used for this experiment, and in SLB, were very high, and would naturally inhibit biosynthesis of proline. As observed, the proline production from Casamino acids took place only after a long incubation period. This delayed production of proline would probably

Table 22. Proline production after 35 days of P. shermanii, P-59, in media containing different amounts of glutamic acid[a]

Glutamic acid added (g/l)	Proline produced (mg/l)
0.0	42.40
0.5	41.85
1.0	41.85
2.0	41.85
4.0	44.95
8.0	38.75

[a]Control medium contains 0.5% Casamino acids.

not occur with glutamic acid because one would not expect that the regulatory site on the enzyme would be inactivated earlier than would the active site. This finding strongly suggests that proline production from Casamino acids is due to arginine catabolism.

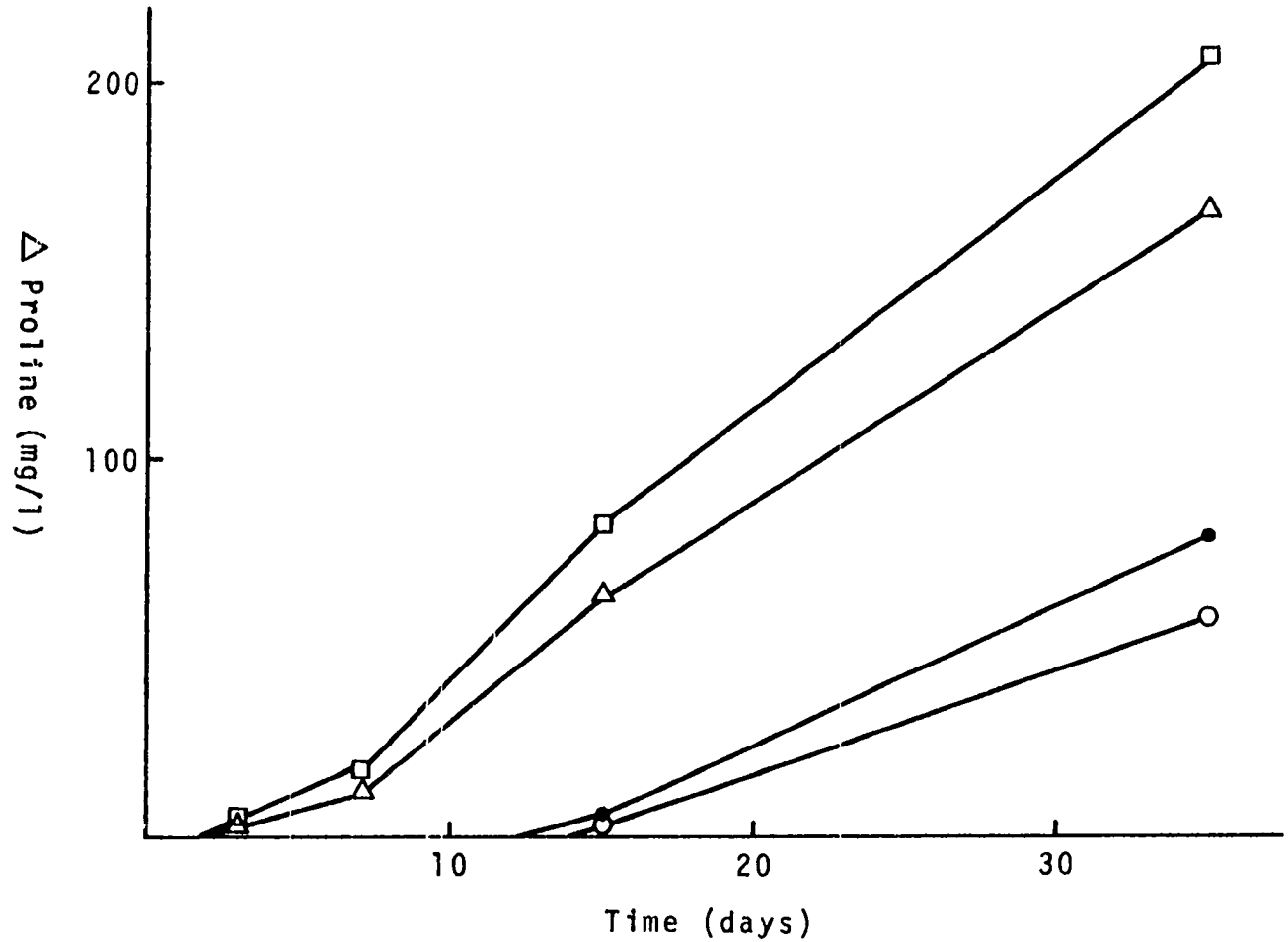
Influence of casein on proline production

Beside peptidase activity and proline biosynthesis, proline may be produced by proteolysis. Virtanen (239) mentioned proteolysis of milk could not be attributed to propionibacteria, indicating that propionibacteria do not have extracellular protease(s). Two experiments were performed, one with 1% Trypticase, and another with 0.5% Casamino acids, plus

the addition of different concentrations of casein. Results from the experiment in which casein was added to Trypticase are shown in Figures 14 and 15. Addition of 0.1% casein did not change the rate of proline production significantly in comparison to the control, but addition of 0.5% casein slowed down the rate. However, after 15-days incubation, the amount of proline produced was the same in the control medium as in the casein-containing media. With extended incubation, the proline content in the casein-containing media continued to increase in contrast to the control. This indicated that proteolysis was taking place in contrast to the findings of Virtanen (239).

Results from the second experiment are presented in Figure 21. Definite proteolysis was taking place, and the proline content increased with increases in the amounts of casein. However, very little proline production took place before 7 days of incubation. In later experiments, it was found that proline production was related to autolysis of the propionibacterial cells. The production of proline due to proteolysis was caused by an intracellular proteinase. Intracellular proteinase is heavily regulated (174), and one of the reasons for the late increase in proline from casein may be that the regulatory system had to be deactivated before the proteinase could act.

Figure 21. Proline production from casein by P. shermanii, P-59, in a defined medium. Symbols: o , Control medium, ● , 0.1% Casein, Δ , 0.5% Casein, □ , 1% Casein


200
100
Δ Proline (mg/l)
10
20
30
Time (days)

Proline distribution

The major reason for conducting this experiment was to see how much proline exists as free amino acids in the amino acid pool of P. shermanii, P-59, as protein-bound proline in the cell, as protein-bound proline in the medium, and as free proline in the medium. The results are shown in Figure 22. One specific remark is necessary. Concentration readings of proline on the different scales of Figure 22 are very different, therefore Table 23 is included to facilitate comparison of proline distribution.

Total cell numbers increased as in the earlier experiments and reached maximum numbers after 3 days of incubation. In this experiment the increase of cell dry weight, as described by Clark et al. (28), was followed. Maximum dry weight of cells was reached after 3 days after which time there was a decrease in weight. It was assumed that 50% of the dry weight is protein (28). The free proline content decreased during the first day, after which it then started to increase, slowly in the beginning, and then faster with time. The largest increase in free proline content occurred between the 4th and the 5th day. These results also concur with those of earlier experiments.

The free proline content in all cells increased paralleling the growth curve, but reached the largest amount on the 4th day, 1 day later than the maximum cell number.

Figure 22. Proline distribution during growth of P. shermanii, P-59, in SLB at 32 C. By proline distribution is meant total free proline, proline in the amino acid pool, total proline in the cells, and proline in released proteins into the medium. Total cell numbers and dry weights of cells also are included in the figure. Symbols: o , Free proline in spent medium, ● , free proline in the amino acid pool, ▲ , Free and protein-bound proline in the bacterial cells, ■, Proline in proteins released by autolysis into the medium, Δ , Cell numbers, □ , Cell dry weight

Free proline in medium (mg/l)
300
400
500
600
Time (days)
1
2
3
4
5
7
8
9
Log 10 number/ml
0
1
2
dry weight of cells (g/l)
1
2
free proline in amino acid pool (mg/l)
20
10
protein-bound proline (mg/l)

Table 23. Proline distribution between cell and medium nitrogen fractions

Proline in N-fraction	Time (days) 1	2	3	4	5
Free proline in medium (mg/l)	279.00	286.75	334.80	437.10	295.10
Free proline in cells (µg/l)	600.00	1680.00	2700.00	2820.00	2520.00
Protein-bound proline in cells (µg/l)	4260.00	8550.00	16272.00	11874.00	10314.00
Protein-bound proline in medium (µg/l)	0.00	0.00	6660.00	22320.00	26040.00
Total proline from cells (µg/l)	4860.00	10230.00	25632.00	37014.00	38874.00
Free proline per cell (µg/cell)	3.8×10^{-7}	3.2×10^{-7}	2.8×10^{-7}	5.2×10^{-7}	7.4×10^{-7}

During the early growth stages, the free proline content/cell decreased up to that point at which the optimum cell number was reached. At the latter stages of growth the free proline/cell increased again. However, the protein-bound proline in the cell paralleled the growth curve and reached the greatest concentration after 3 days, after which it decreased. No protein-bound proline was found in the medium before the 3rd

day, and the most rapid increase took place up to the 4th day, after which the rate decreased. This agreed with results from later experiments that showed that the most rapid autolysis generally took place at this time period. However, the rate of decrease in protein-bound proline in the cells, and the rate of increase in protein-bound proline in the medium, indicated that a high number of new cells also were being produced at this stage. So, an equilibrium existed between autolysis of old cells and the production of new cells.

Contributions of proline from these fractions to the total free proline in the medium can only be minimal. Even if the protein released into the medium was hydrolyzed by the intracellular proteinase, it would contribute only about 35 to 40 mg/l.

Conclusion

P. shermanii, P-59, produces proline in great amounts from peptide-containing media. Enzymatic casein hydrolysates are the best substrates. Meat hydrolysates are less effective substrates, and, by adding Peptone to a Trypticase-containing medium, proline production from Trypticase is inhibited. The mechanism by which proline is produced is complex. The importance of the different possibilities for propionibacterial proline production is as follows:

1. Peptidase activity is definitely the most important factor in proline production.
2. Some proline can be produced by proteolysis, but the production rate is slower, and is of less importance than peptidase activity.
3. Proline biosynthesis from glutamic acid is of little importance, because of feed-back inhibition.
4. Proline production from arginine probably occurs but would be of little importance in comparison to peptidase activity.
5. Cellular proline would be of little importance. The release of protein into the medium, however, coincides with proline production indicating that proline is produced by intracellular enzymes.

This production of proline could occur in cheese, because a continuous increase in peptides takes place during cheese ripening (180). This continuing proteolytic activity would furnish the propionibacteria with the necessary substrates for proline production.

Proline Production by Strains of Propionibacteria, and the Effect of Conditions Related to Cheese Ripening

Strain differences

The experiments concerning the mechanisms of propionibacterial proline production, were carried out using P. shermanii, P-59. In Swiss cheese manufacture, however, different species and strains of propionibacteria are used. The following experiment was performed to observe if different species and strains of propionibacteria would show differences in rate of proline production. The amounts of proline produced after 15 days are shown in Figure 23, and the average values of proline produced from the strains within each species after 15 and 35 days are shown in Table 24.

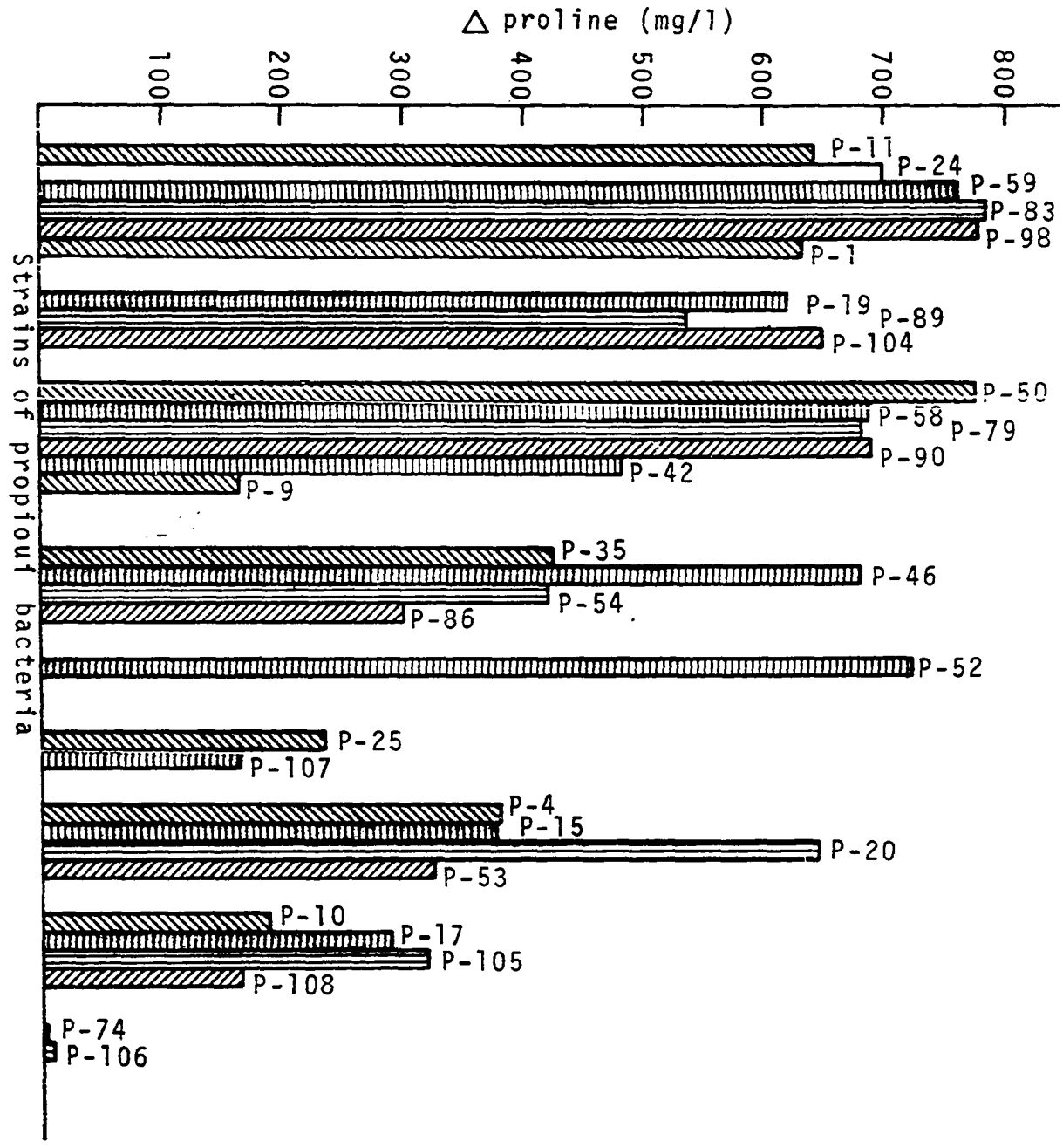
Large differences in proline production were observed between species and between strains within species. P. shermanii produced the highest amount of proline after 15 days, especially strains P-24, P-59, P-83, and P-98. A single strain of P. jensenii produced about the same amount of proline as the average P. shermanii. P. freudenreichii, which is closely related to P. shermanii (129), developed smaller amounts of proline than P. shermanii. Very little strain difference was observed. The proline content in cultures of P. pentosaceum was about the same as for

Table 24. Average amounts of free proline produced after 15 and 35 days by different species of propionibacteria

Species	Time (days) 15	35
	Proline (mg/l)	
P. freudenreichii	606.65	894.95
P. intermedium	0.00	659.53
P. jensenii	723.85	895.90
P. pentosaceum	580.55	785.13
P. raffinosaceum	200.73	777.33
P. rubrum	223.35	769.58
P. shermanii	714.95	896.68
P. thoenii	430.73	829.25
P. zeae	456.86	865.39

P. freudenreichii but there were large strain differences in this species. P. pentosaceum, P-9, earlier classified as P. arabinosum, P-9, which is often used in Swiss cheese manufacture, produced only about 170 mg proline/l after 15 days compared to 580.55 mg/l (the average for all strains of P. pentosaceum). The proline concentration developed by P. zeae was definitely lower, however, one strain, P-46, produced very high amounts after 15 days. P. thoenii produced, on an average 430 mg proline/l which was higher than the amounts

Figure 23. Proline production by 32 strains of the genus Propionibacterium at 32 C for 15 days


Δ proline (mg/l)
100
200
300
400
500
600
700
800
Strains of propiout bacteria
P-11
P-24
P-59
P-83
P-98
P-1
P-19
P-89
P-104
P-50
P-58
P-79
P-90
P-42
P-9
P-35
P-46
P-54
P-86
P-52
P-25
P-107
P-4
P-15
P-20
P-53
P-10
P-17
P-105
P-108
P-74
P-106

produced by P. rubrum. Only one strain of P. thoenii, P-20, produced definitely higher amounts of proline than the other strains. The proline content after 15 days from P. raffinosaceum was only 200 mg/l, and P. intermedium produced no proline after 15 days.

After continued incubation for 35 days, the differences in proline production between the various species became much smaller, and it was impossible to differentiate between different species. Only P. intermedium showed a definitely lower proline content, but it still produced 659 mg/l, which would have been a large amount of proline had it been produced in 15 days. The variation in proline production after 15 days between different species was probably caused by rate differences. Another factor that could be involved was that variances in maximum cell numbers correlate with proline production. P. shermanii, P-59 and P-98, however, produced about the same amount of proline in 15 days. Maximum cell numbers were, respectively, 60×10^8 and 30×10^8. Even larger dissimilarities in cell numbers were found between P. thoenii, P-15 (19×10^8), and P-4 (4.4×10^8), while the proline content after 15 days was about the same. Differences in the rate of proline production were probably caused by variations in the rate of autolysis, which will be discussed in greater detail in a later part of this thesis. Autolysis

releases intracellular enzymes which will hydrolyze peptides in the medium. Variation in proline production was probably more closely related to the rate of release of these enzymes into the medium, rather than to large differences in specific activities.

The rate of proline production seemed to be species determined, even if some large strain differences were observed and could possibly be used as an taxonomic tool in addition to other characters (129, 152). Rate of autolysis, however, may possibly be a better and a faster way to measure these dissimilarities.

After considering the results of this experiment the following strains were chosen: P. freudenreichii, P-19, P. pentosaceum, P-9, P. shermanii, P-1, P-24, and P-59, and P. zeae, P-35. These strains were used in a few selected experiments to observe strain differences in proline production under other conditions. All of these strains have been used in Swiss cheese manufacture.

Influence of temperature on proline production by P. shermanii, P-59

Previous experiments had been done at 32 C, the optimum growth temperature for P. shermanii, P-59. However, Swiss cheese is ripened at 21 C, and the proline-producing capacity may change quite radically at this lower temperature. It was

earlier stated that propionibacteria could grow at temperatures between 15 and 40 C (156), however, Park et al. (157) found that these bacteria could grow at 10 C and lower. In this experiment, however, rather rapid growth was wanted so a temperature range of 15 to 37 C was used.

Maximum cell numbers and pH changes in SLB are reported in Table 25. The maximum numbers at 37, 32, and 27 C were reached after 2 days, at 21 C after 5 days, and at 15 C after 15 days. These differences in growth rates also were reflected in pH changes which were slower at temperatures lower than 27 C, especially at 15 C.

Rate of proline production varied with the different growth temperatures as shown in Figure 24 and optimum production took place at 32 C (Figure 25). The most rapid increase in proline occurred at 32 C while the gains in proline content at 27 and 37 C were slightly slower. Proline accumulation at 21 C was definitely slower and did not start before 7 days, in contrast to only 2 to 3 days at the higher temperatures. At 15 C, no increase in proline was observed before 35 days.

Another effect which became more apparent in this experiment, was that the free proline content decreased during the lag and the logarithmic growth phase of the P. shermanii strain. This decrease in proline took place during the first 2 days at the higher temperatures, but at 21 C it lasted for

Table 25. Effect of growth temperature on maximum cell numbers and pH changes by Propionibacterium shermanii, P-59, in Sodium lactate broth

Temperature (centigrade)	Maximum cell numbers/ml ($x10^6$)	Time (days)		
		8	15	35
		pH[a]		
15	43,000	6.68	6.58	7.20
21	76,000	6.30	7.08	7.30
27	78,000	6.50	7.18	7.30
32	75,000	6.30	7.20	7.30
37	18,000	6.62	7.10	7.18

[a]Initial pH of Sodium lactate broth is pH 6.8.

Figure 24. Influence of different incubation temperatures on proline production from SLB by P. shermanii, P-59. Symbols: ● , 15 C, ▲ , 21 C, ▫ , 27 C, o , 32 C, △ , 37 C

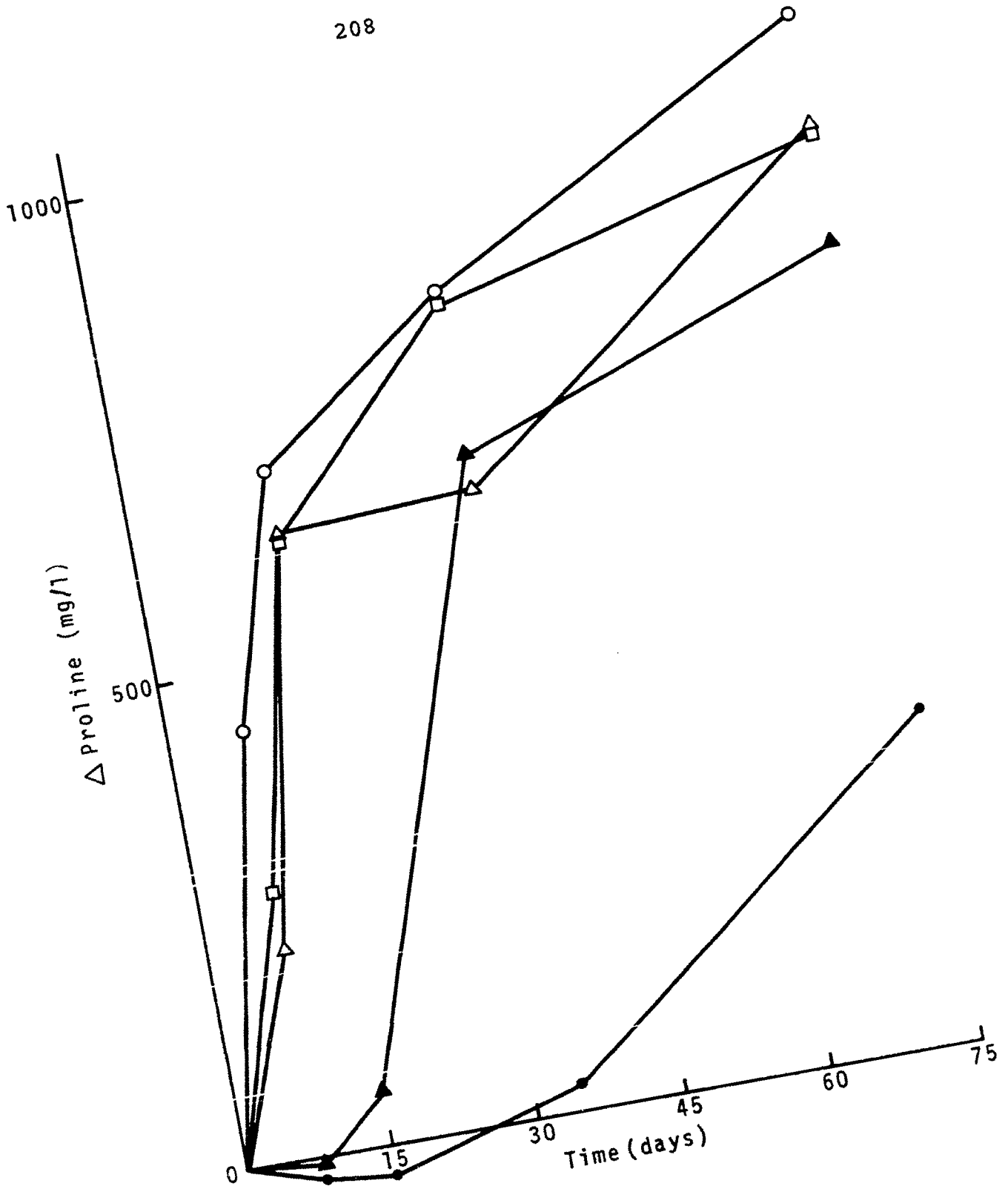
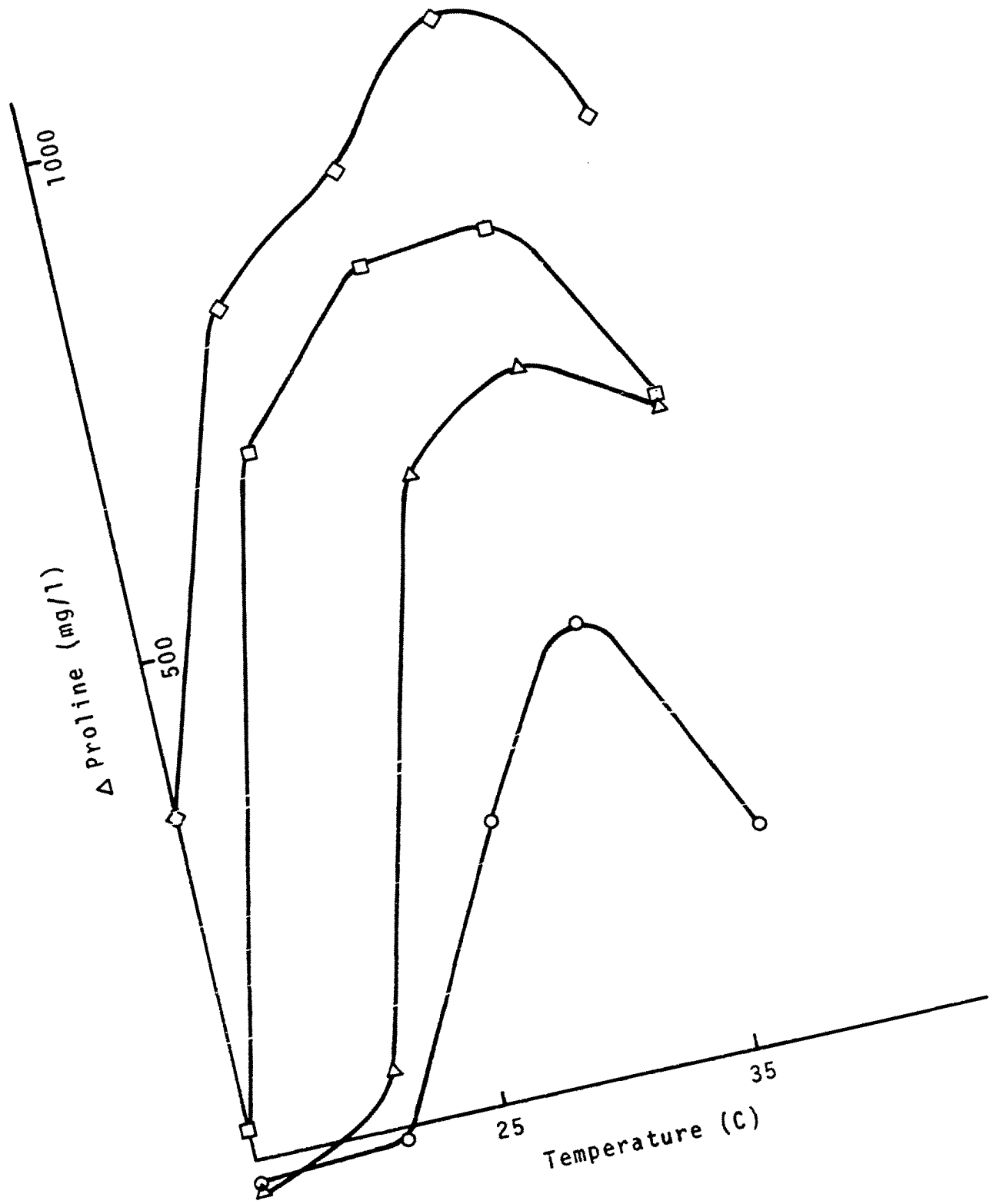

1000
500
0
Δ Proline (mg/l)
15
30
45
60
75
Time (days)

Figure 25. Influence of temperature on proline production from SLB by P. shermanii, P-59. Symbols: o , 8 days, Δ , 15 days, □ , 35 days, ◇ , 75 days


1000
500
Δ Proline (mg/l)
25
35
Temperature (C)

7 days, and at 15 C for about 25 days. In later experiments, the increase in proline content was found to be related to autolysis which is an enzymatic process. Autolysis was probably most rapid at 32 C or higher, as indicated by the rapid increase in free proline at these temperatures. The increase in proline at 37 C was about the same as for 32 C, even though the cell numbers were much lower, which may indicate faster autolysis. Proline content also started to increase at about the same time maximum cell numbers were reached. At the lower temperatures, the increase in proline started 3 and 10 days after maximum cell numbers were reached which may indicate a slower autolysis.

Proline production at 3 and 21 C for six strains of propionibacteria

Large differences in rate of proline production were observed between different species and strains of propionibacteria. However, 32 C, the optimum temperature for growth, is much higher than the temperature in the warm room (21 to 25 C) and in the curing cellar (2 to 5 C) (188). The next step was to observe proline production by propionibacteria in SLB at 3 and 21 C. Six strains, commonly used in Swiss cheese manufacture, were used in these experiments. The strains were selected because of their capacity to grow at low temperatures (278). P. pentosaceum, P-9, grew at 15 C,

P. shermanii, P-1, at 10 C, P. freudenreichii, P-19, P. shermanii, P-24, and P. zeae, P-35, at 6.8 C, and P. shermanii, P-59, at 3.8 C (78).

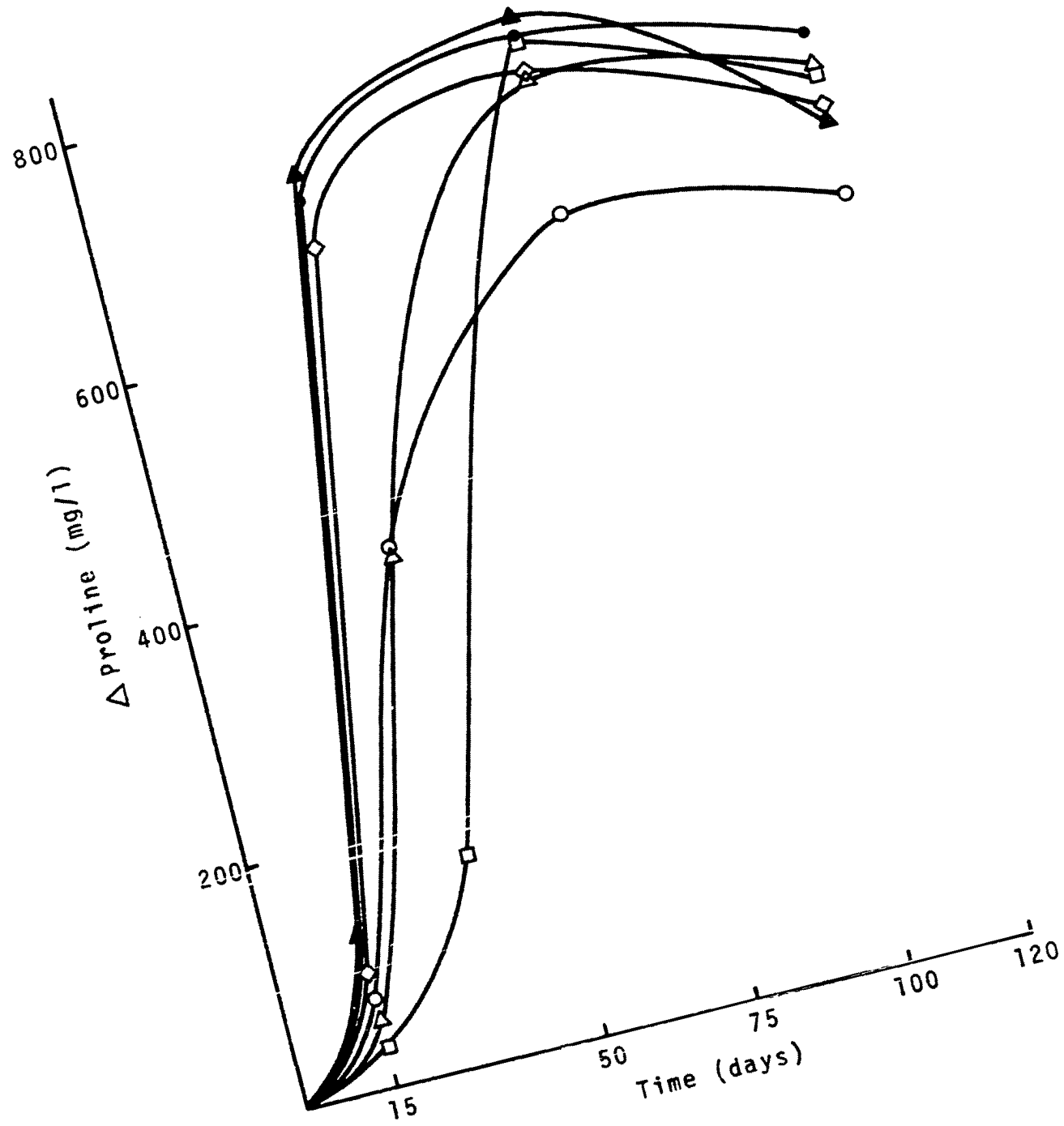
Proline production at 21 C from Trypticase was examined first. Maximum cell numbers and pH changes are shown in Table 26. Some variation was observed in total cell numbers

Table 26. Maximum cell numbers and pH changes by six different strains of propionibacteria at 21 C

Strains of propionibacteria	Maximum cell numbers/ml x 10^5	Time (days) 6 pH	Time (days) 15 pH
P. pentosaceum, P-9	6,000	6.58	7.00
P. shermanii, P-1	69,000	6.50	6.80
P. zeae, P-35	47,000	6.58	6.90
P. freudenreichii, P-19	73,000	6.50	6.80
P. shermanii, P-24	87,000	6.54	6.96
P. shermanii, P-59	61,000	6.45	6.85

between the different strains used, but these differences were not very significant, except with P. pentosaceum, P-9, where growth was about a log number less than the others. pH changes were about the same for all strains. Proline production by the different strains is shown in Figure 26. The most rapid increases in proline were found with

Figure 26. Proline production by six strains of *Propionibacterium* from SLB at 21 C. Symbols: o , *P. pentosaceum*, P-9, Δ , *P. shermanii*, P-1, □ , *P. zeae*, P-35, ◇ , *P. freudenreichii*, P-19, ● , *P. shermanii*, P-24, ▲ , *P. shermanii*, P-59


800
600
400
200
Δ Proline (mg/l)
15
50
75
100
120
Time (days)

P. shermanii, P-59, and P-24, which showed no significant differences, followed by P. freudenreichii, P-19. All of these strains were capable of growing at 6.8 C. P. pentosaceum, P-9, and P. shermanii, P-1, exhibited the same rate of proline production during the first 35 days, but later relatively higher amounts of proline were brought about by P. shermanii, P-1. P. zeae, P-35, showed the lowest rate of proline production, even though it could grow at 6.8 C. The rate of proline increase was somewhat related to their minimum growth temperature, but, in addition, their rate of autolysis for each strain would influence proline production.

Generally, a high amount of flavor develops in the Swiss cheese in the curing cellar (122). If proline is important for Swiss cheese flavor, proline content would be expected to increase at 3 C. The same strains as were used for the last experiment were used. These organisms did not grow at this temperature. Therefore, they were incubated at 32 C for 6 days to reach high cell numbers and to induce autolysis before they were cooled down and incubated at 3 C. The results for maximum cell numbers after 3 days and pH changes are shown in Table 27. The numbers of P. pentosaceum, P-9, and P. zeae, P-35, were somewhat lower than for the other strains. The pH changes, which are toward the basic side, indicated metabolic activity in all strains used at 3 C.

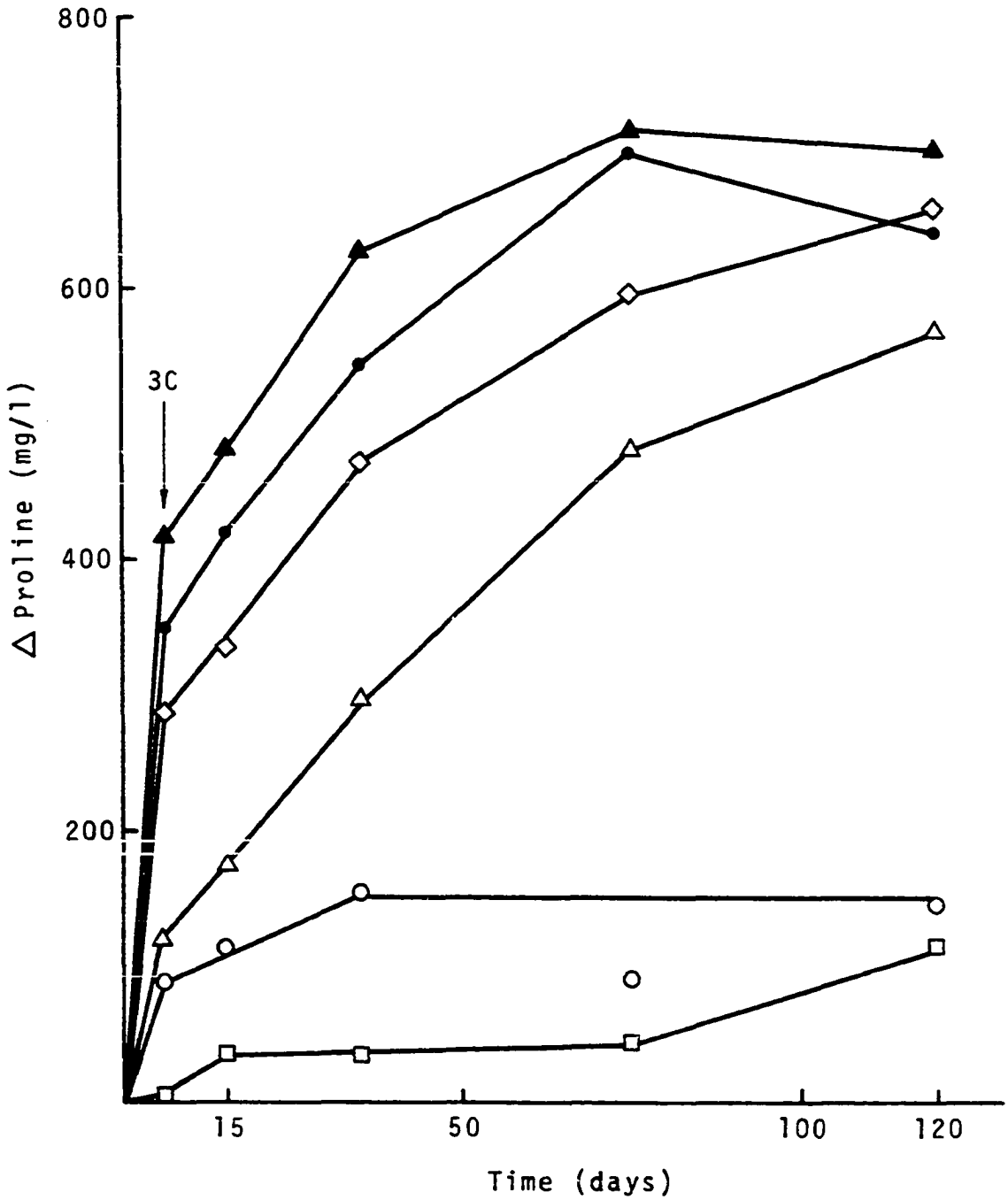
The results for proline production from Trypticase are

Table 27. Maximum cell numbers and pH changes by six different strains of propionibacteria at 3 C

Strains of propionibacteria	Maximum cell numbers/ml x10^5	Time (days) 3	6	15
P. pentosaceum, P-9	20,000	6.30	6.25	6.75
P. shermanii, P-1	75,000	6.50	6.60	6.50
P. zeae, P-35	37,000	6.52	6.45	6.75
P. freudenreichii, P-19	65,000	6.60	6.30	6.70
P. shermanii, P-24	54,000	6.50	6.52	6.60
P. shermanii, P-59	54,000	6.60	6.55	6.75

shown in Figure 27. After 6 days, the largest amount of proline was made by P. shermanii, P-59, followed by P. shermanii, P-24, and P. freudenreichii, P-19. P. shermanii, P-1, and P. pentosaceum, P-9, produced quite small amounts of proline, and P. zeae, P-35, showed almost no increase. At 3 C, the proline content continued to increase quite rapidly for all strains except P. pentosaceum, P-9, and P. zeae, P-35, with which only a slow accumulation of proline took place. A logical explanation centers on the degree of autolysis present after 6 days. A discussion of autolysis is presented in detail in a later section. The most proline was produced by P. shermanii, P-59, and P-24, after 75 days, after which a

Figure 27. Proline production by six strains of Propionibacterium from SLB at 3 C. The inoculated media were incubated at 32 C for 6 days before they were transferred to a 3 C incubator. Symbols: ○, P. pentosaceum, P-9, △, P. shermanii, P-1, □, P. zeae, P-35, ◇, P. freudenreichii, P-19, ●, P. shermanii, P-24, ▲, P. shermanii, P-59


800
600
400
200
3C
Δ Proline (mg/l)
15
50
100
120
Time (days)

decrease in proline took place. This experiment constituted a dynamic system, so while proline was produced by peptidases, some proline also was being degraded.

The results of this experiment show that proline could be produced fairly rapidly at 21 C, and that a continued increase took place at 3 C. These temperatures are, as mentioned earlier, similar to the temperatures in the warm room and in the curing cellar, so proline could be produced during cheese making conditions. The differences in proline production between the different strains and species also confirms the results of Hettinga and Reinbold (77), that propionibacteria which could grow at 3.8 and 6.8 C, generally show greater metabolic activity than those growing at 10 and 15 C.

Influence of initial pH on proline production

The pH of Swiss cheese is generally about pH 5.2 when it is moved into the warm room. A rapid increase occurs in the warm room and the pH will be about 5.6 when the cheeses are moved into the curing cellar (51). Different pH values may influence the proline-producing capacity of the propionibacteria, and the next experiment describes proline production by P. shermanii, P-59, from SLB with different initial pH values.

The maximum cell numbers showed little variation with initial pH, and maximum cell numbers were reached at the same time. The results related to pH changes and proline production are shown in Figure 28. The pH change in the medium with an initial pH of 6.8 was the only one showing a decrease during the first 2 days followed by a continuous increase up to pH 7.8. With an initial pH of 6.0, the increase in pH was continuous during the entire experiment and the final pH after 35 days was pH 7.3. However, with an initial pH of 5.2 or 5.6, a rapid increase was observed during the first 3 days, and, thereafter, pH remained constant, at, respectively 5.9 and 6.05. The increase in pH from pH 5.2 parallels the increase in pH in the cheese in the warm room, however, the increase in pH is less in the cheese, probably because the cheese is more heavily buffered.

The initial pH did influence the rate of proline production. A slower rate was observed at the lower pH values, but after 35 days total amounts produced were the same, except for the trial with an initial pH of 6.0 which produced slightly higher amounts of proline. From this, it can be concluded that high amounts of proline could be produced at pH values found in Swiss cheese in the warm room and in the curing cellar. The rate differences were probably caused by two factors; the variation in enzymatic activity of the proline-producing enzymes with pH, and the variation in

Figure 28. Influence of initial pH of SLB on proline production and pH changes by P. shermanii, P-59. (——) proline production, (----) pH changes. Symbols: o , pH 6.8, ● , pH 6.0, Δ , pH 5.5, ▲ , pH 5.2

Proline (mg/l)
200
400
600
10
20
30
Time (days)
5
6
7
8
pH

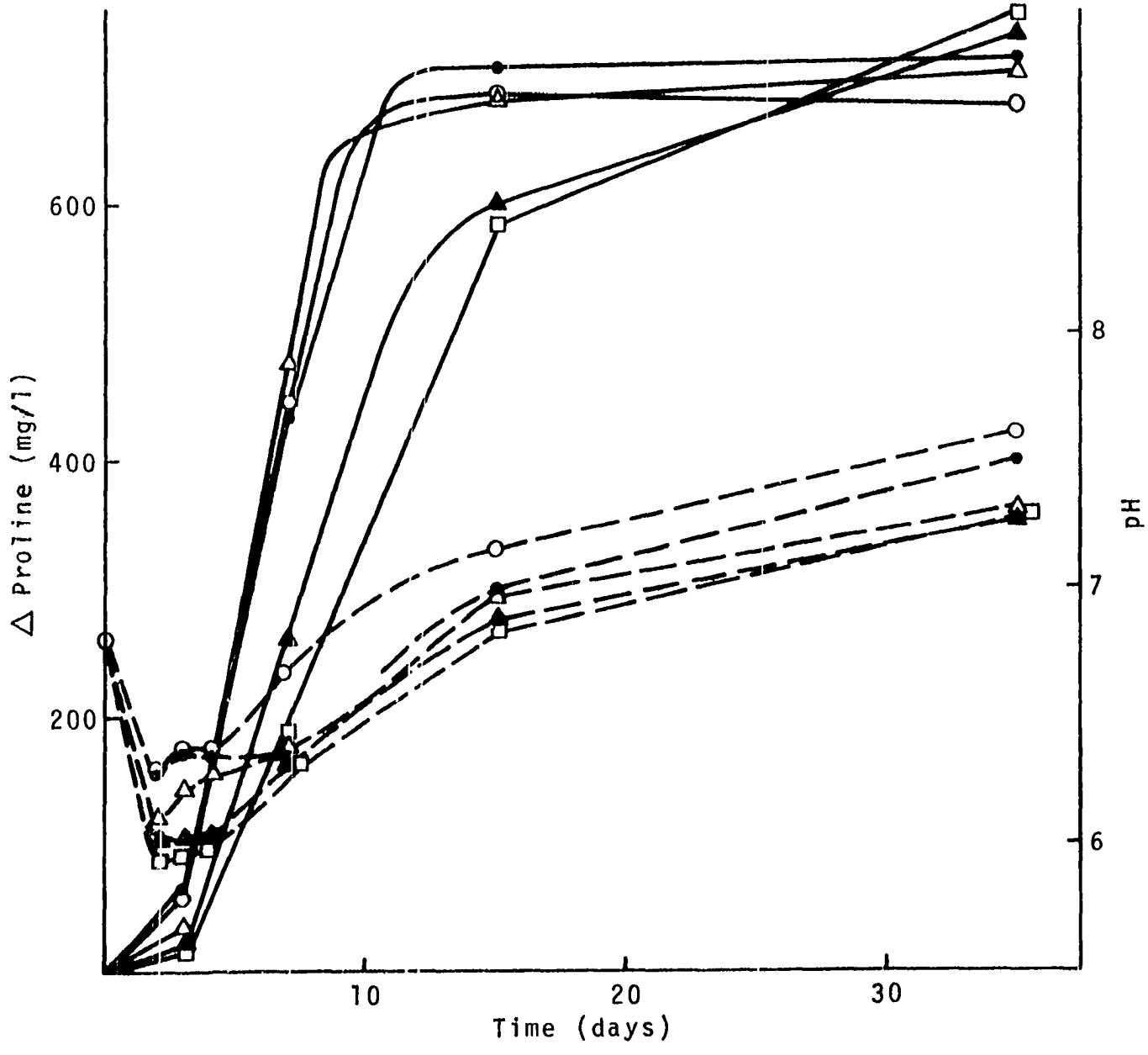
autolysis with pH.

Influence of NaCl on proline production

Swiss cheeses are salted to improve flavor and to control ripening. Propionibacteria are usually very sensitive to salt; therefore, Swiss cheese must be salted lightly if eyes are to develop normally (51). About 3% NaCl was shown by Antila (1) to be necessary to reduce growth rate, but, generally, the salt concentration is about 1% in Swiss cheese (188).

In this experiment, SLB with salt concentrations from 0.1 to 1.5% was used. About the same cell numbers and the same growth rate was observed for P. shermanii, P-59. However, the pH changes varied with salt concentrations as shown in Figure 29. During the first 2 days the decrease in pH increased with increasing amounts of NaCl, and this led to lower pH values after 35 days than the control without salt. NaCl concentrations of 0.1 and 0.5% had very little, if any, effect on the rate of proline production as shown in Figure 29, except that slightly higher amounts were produced after 35 days with NaCl addition. With higher contents of NaCl the proline production rate was definitely decreased, with the least proline produced at 1.5% NaCl. However, the final content of proline after 35 days was higher than that in the control. The results indicate that the rate of proline production is slowed down by increasing salt concentrations.

Figure 29. Influence of NaCl in SLB on proline production and pH changes by P. shermanii, P-59. (——) proline production, (----) pH changes. Symbols: o , 0% NaCl, ● , 0.1% NaCl, Δ , 0.5% NaCl, ▲ , 1% NaCl, □ , 1.5% NaCl


600
400
200
Δ Proline (mg/l)
8
7
6
pH
10
20
30
Time (days)

This may be caused by changes in the optimum conditions for the proline-producing enzymes through differences in pH and salt concentrations, or, more probably, by changes in ionic strength which will influence the autolysis, as discussed in a later experiment.

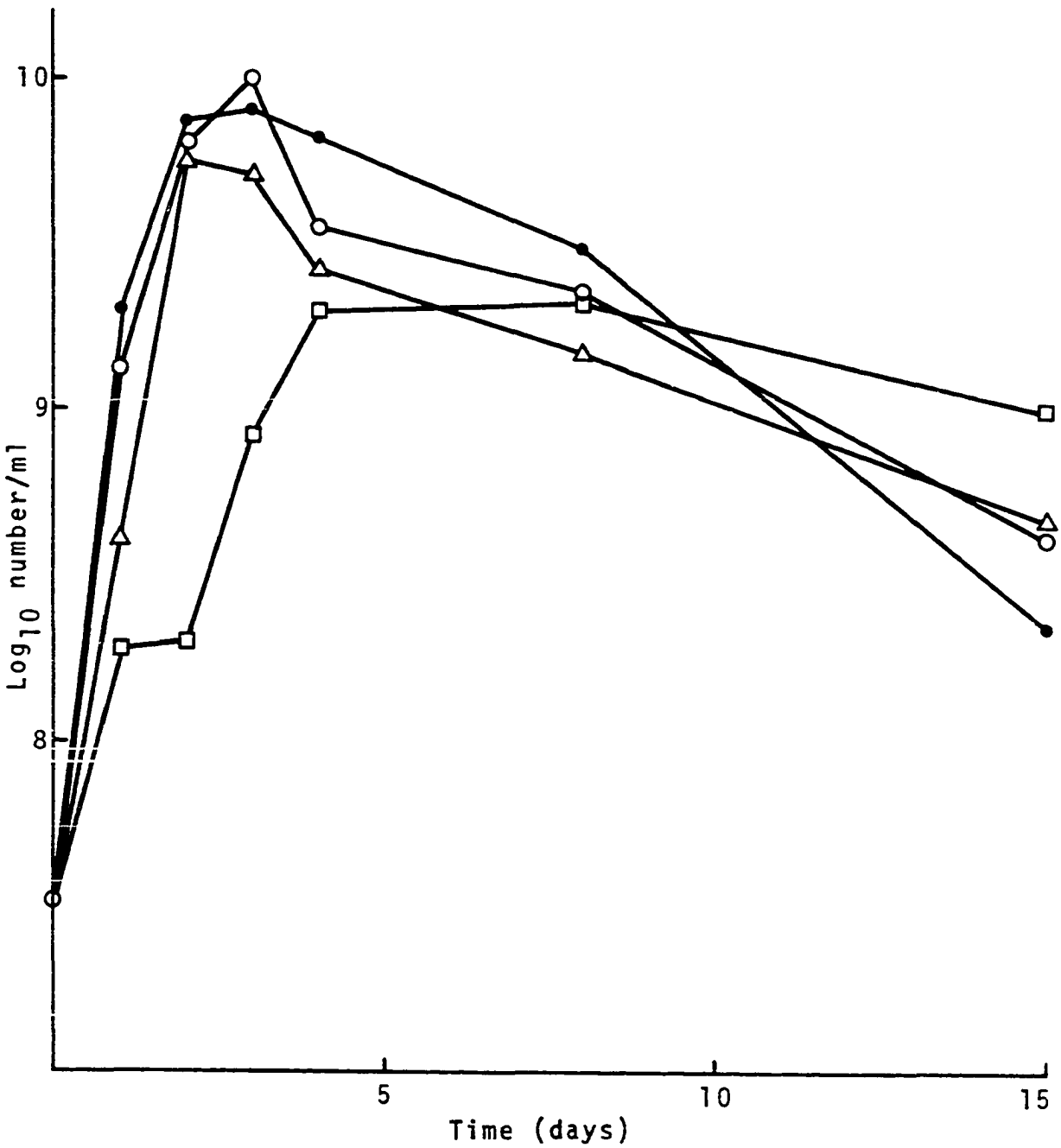
Influence of copper on proline production

Originally, Swiss cheese was made in copper kettles, but now, at least in the U.S., Swiss cheese is made exclusively in stainless steel vats. Propionibacteria were found to be inhibited by copper, and copper also retarded the formation of propionic acid, while little influence was found on proteolysis (135). The question is, does copper have any influence on proline production by P. shermanii, P-59, in SLB and in milk?

Eight and 16 ppm Cu^{++} were added to the basal media. Growth of P. shermanii, P-59, under these conditions is shown in Figure 30. Addition of 8 ppm Cu^{++} led to a slower growth rate and lower cell numbers. Sixteen ppm Cu^{++} had a strong inhibitory effect. The growth rate was much slower, a much lower number of cells was found, and maximum cell numbers were reached after 4 to 5 days in comparison with 2 to 3 days for the control medium and the medium containing 8 ppm Cu^{++}.

Proline production was somewhat retarded at 8 ppm Cu^{++},

Figure 30. Influence of copper and calcium carbonate on growth of P. shermanii, P-59, at 32 C, in SLB. Symbols: o , Control, SLB, Δ , 8 ppm Cu^{++}, □ , 16 ppm Cu^{++}, ● , 1% $CaCO_3$


10
9
8
Log10 number/ml
5
10
15
Time (days)

but only slightly; after 35 days, the amount of proline was the same (Figure 31) as for the control. However, at 16 ppm CU^{++}, significantly slower proline production took place, but after 75 days the amount produced was about the same as in the control.

When the same experiment was performed using milk fortified with 0.1% sodium lactate, 0.1% Trypticase, and 0.1% yeast extract, very little difference in growth was observed with addition of copper (Figure 32). Somewhat slower growth rates were observed with increasing amounts of copper, but the differences were slight. When proline production was measured, no difference was observed between samples containing 8 and 16 ppm Cu^{++} as shown in Figure 33. However, more proline was utilized during the initial 2 days in copper-containing media, and then the increase was parallel with the control up to the 15th day.

The effect of copper on the growth of propionibacteria was quite different in SLB and in milk. In SLB the growth was very strongly retarded by 16 ppm Cu^{++}, which confirmed Maurer's results (135), while no effect was observed in milk with the same copper concentration. This is probably caused by the fact that most of the added copper is associated with the milk proteins (194). The difference in proline production by P. shermanii, P-59, in SLB and SLB containing 16 ppm Cu^{++} was probably caused more by difference in growth than by

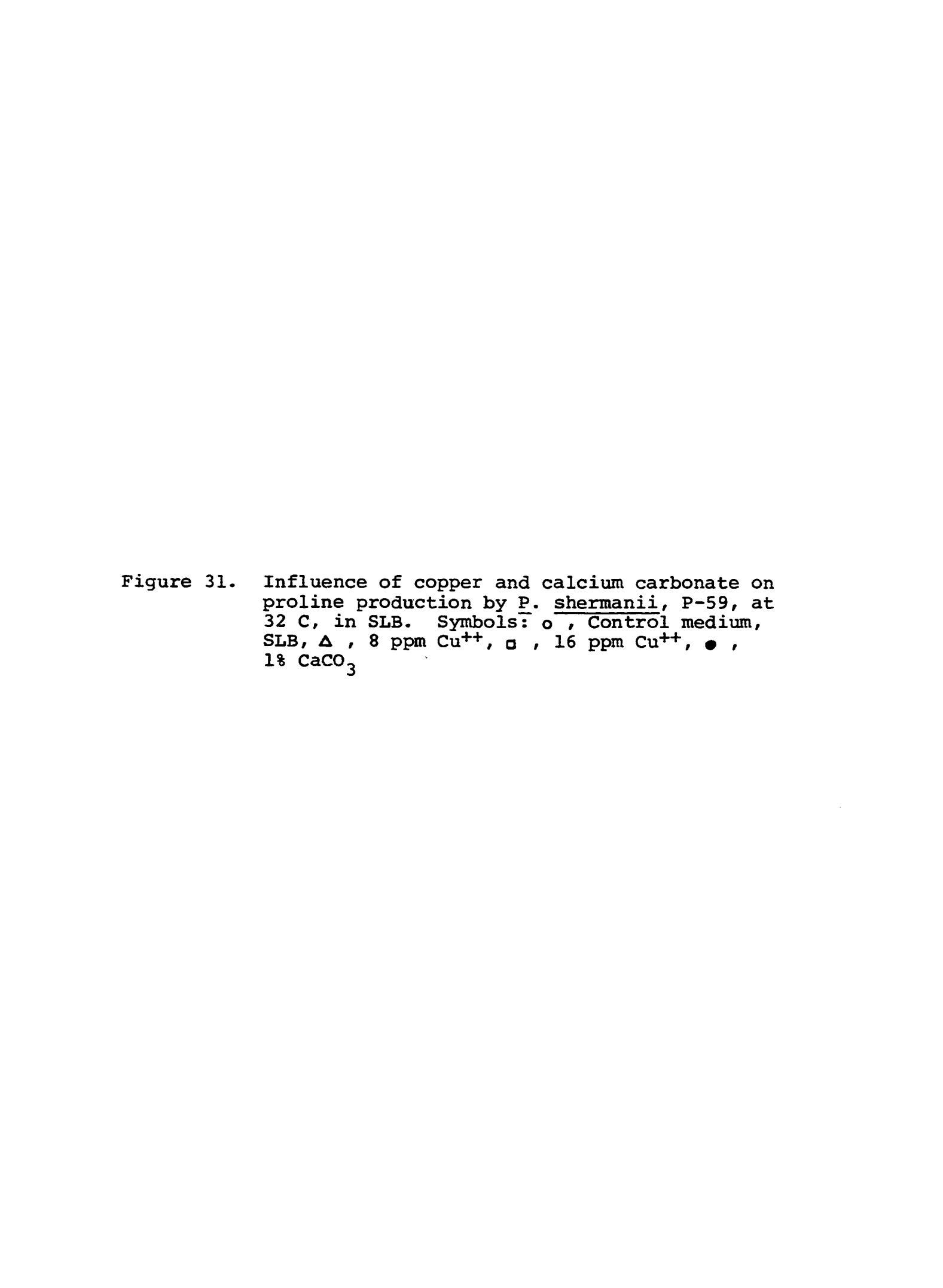


Figure 31. Influence of copper and calcium carbonate on proline production by P. shermanii, P-59, at 32 C, in SLB. Symbols: o , Control medium, SLB, Δ , 8 ppm Cu^{++}, □ , 16 ppm Cu^{++}, ● , 1% $CaCO_3$

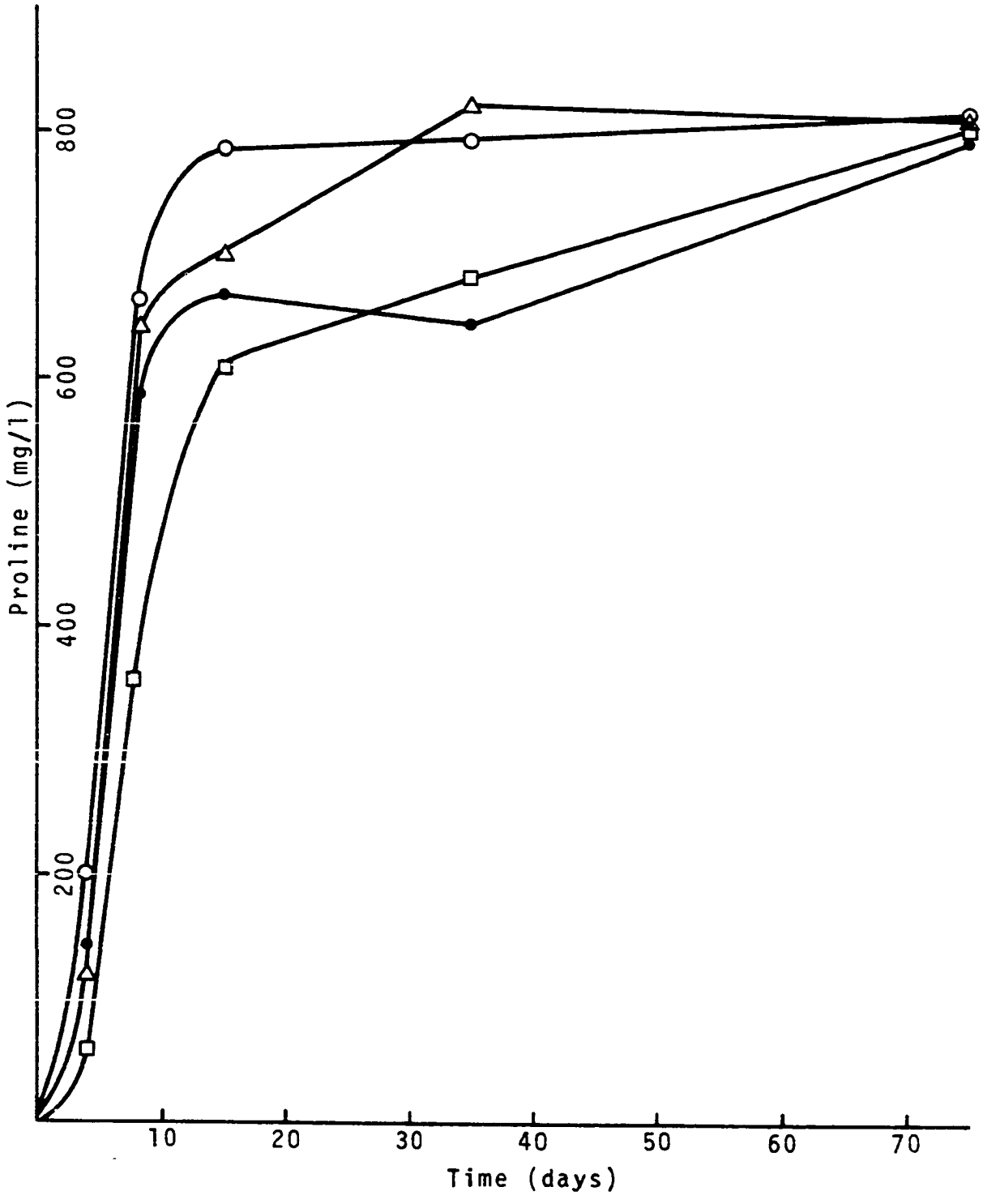

Proline (mg/l)
800
600
400
200
10
20
30
40
50
60
70
Time (days)

Figure 32. Influence of copper on growth of P. shermanii, P-59, at 32 C, in fortified reconstituted milk. Symbols: □ , 0.1 sodium lactate, 0.1% Trypticase, 0.1% yeast extract, ● , 0.1% sodium lactate, 0.1% Trypticase, 0.1% yeast extract, and 8 ppm Cu^{++}, ▲ , 0.1% sodium lactate, 0.1% Trypticase, 0.1% yeast extract, and 16 ppm Cu^{++}

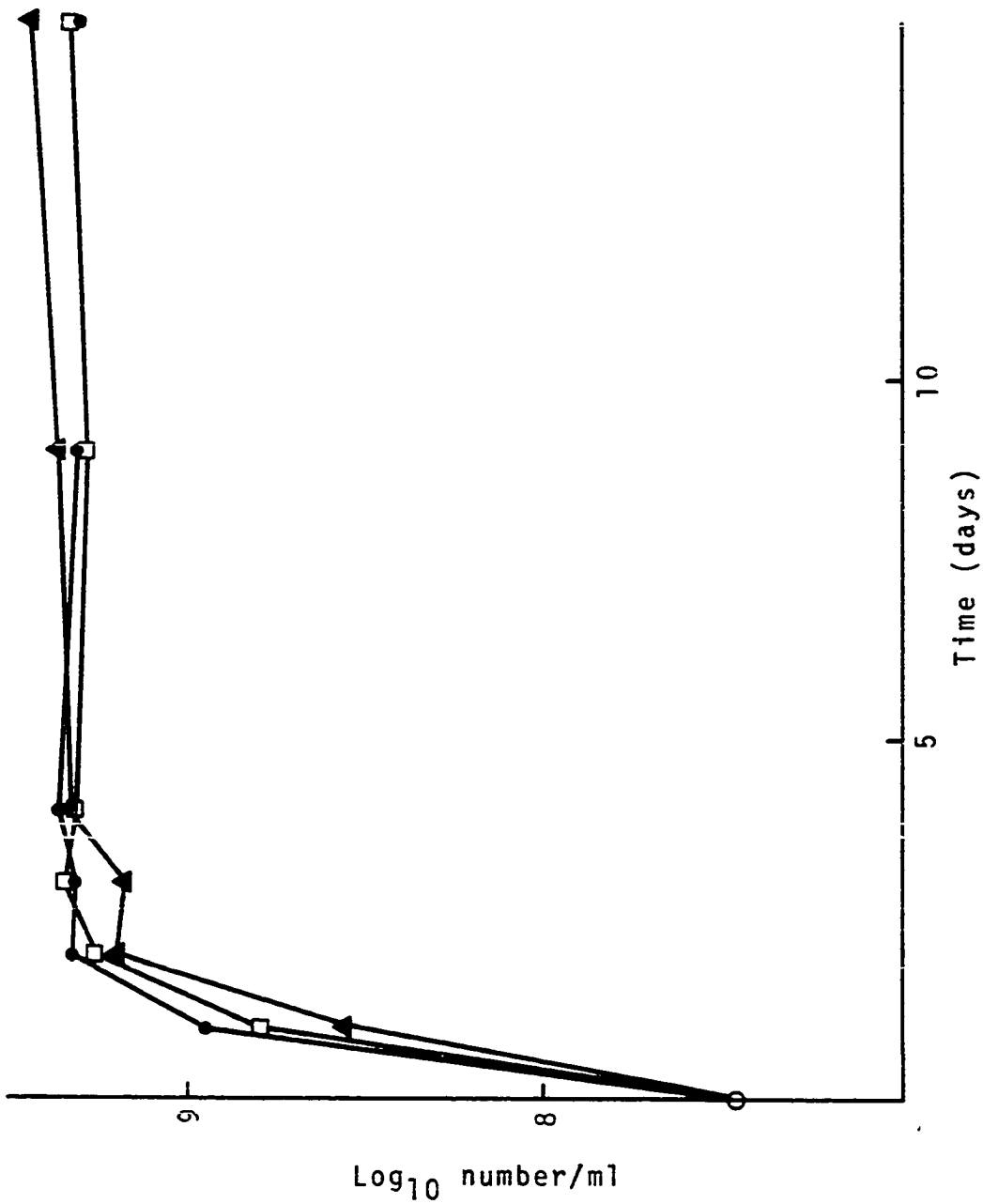
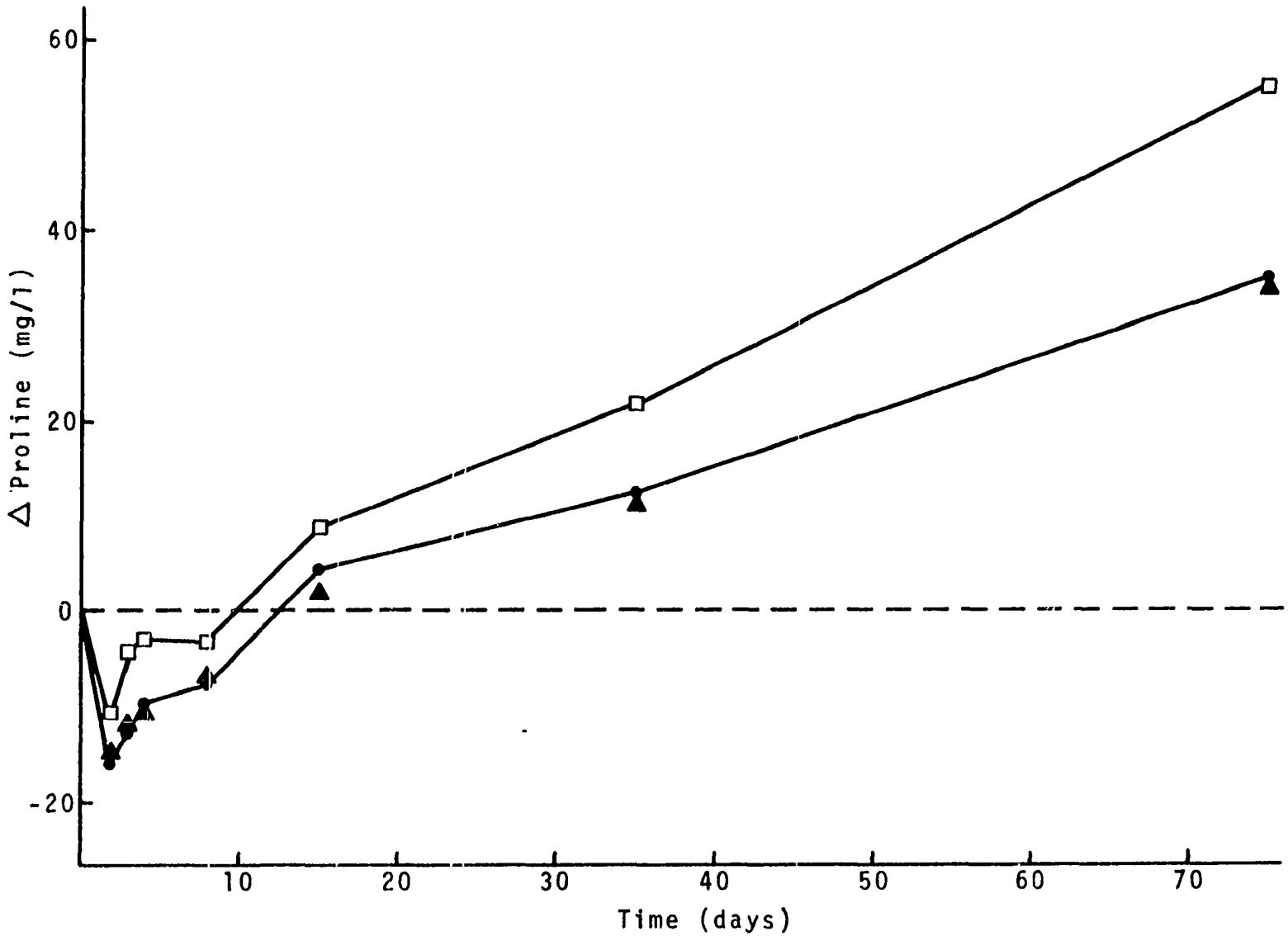

9
8
Log10 number/ml
5
10
Time (days)

Figure 33. Influence of copper on proline production by P. shermanii, P-59, at 32 C, in fortified reconstituted milk. Symbols: □ , 0.1% sodium lactate, 0.1% Trypticase, 0.1% yeast extract, ● , 0.1% sodium lactate, 0.1% Trypticase, 0.1% yeast extract, 8 ppm Cu^{++}, ▲ , 0.1% sodium lactate, 0.1% Trypticase, 0.1% yeast extract, 16 ppm Cu^{++}


60
40
20
0
-20
Δ Proline (mg/l)
10
20
30
40
50
60
70
Time (days)

differences in enzyme activity. However, low amounts of copper retards proline production in milk, especially at later stages, which also may be an indication of a second enzyme, perhaps a protease, which is retarded by copper.

Conclusion

Large differences in the rate of proline production by different species and strains within species were shown. P. shermanii, P-59, a fast proline-producer, and P. pentosaceum, P-9, a slow proline-producer, are both used in Swiss cheese manufacture. Flavor development by P. shermanii, P-59, however, is faster than for P. pentosaceum, P-9, and selection of a fast proline-producer may shorten the time needed for full flavor development.

All strains of propionibacteria tested produced proline at 3 and 21 C, but large strain differences were observed. These variations were related to growth rates and rate of autolysis. Selection of propionibacteria that can grow and produce proline at low temperatures may improve the rapidity of the flavor development. The proline production by propionibacteria at pH values and NaCl concentrations found in Swiss cheese was retarded, but the final amount produced was the same as in the control. Copper affected the growth, but not the proline production. This shows that propionibacteria can produce proline in cheese if the required

precursors are present.

Autolysis and Proline Production

Proline imino- and imidopeptidase and other peptidases

Peptidases have been stated to be the most important cause of proline production. The hypothesis thus becomes: intracellular enzymes released by autolysis produce proline. In this section, the relation of autolysis to proline production and release of proline-producing enzymes were measured.

The presence of proline iminopeptidase which acts on peptides with proline at the amino end, and proline imidopeptidase which acts on peptides with proline at the carboxyl end was determined. L-Prolyl-glycine (L-Pro-Gly) and glycyl-L-proline (Gly-L-Pro) were used as substrates. Later, a larger number of prolyl peptides were assayed to measure the effect of other side groups on enzyme activity. A few other peptides also were assayed to determine if other peptidases were present. Supernatant from P. shermanii, P-59, was used for this experiment.

It has been observed that certain metal ions activate the enzyme (162), so the effect of Co^{++}, Mn^{++}, and Na^{+} on enzyme activities was measured. The results are shown in Table 28. High activities were observed with L-Pro-Gly as substrate, but low activities were found with Gly-L-Pro. Both Co^{++} and Mn^{++}

Table 28. Effect of Co^{++}, Mn^{++}, and Na^{+} on enzyme activities on L-Pro-Gly and Gly-L-Pro in phosphate buffer (pH 7.0)[a]

Salts added	Enzyme activities (mg Pro/ml supernatant/h)	
	Pro-Gly	Gly-Pro
None	600.0	15.2
Co^{++}	1045.0	60.5
Mn^{++}	826.0	12.5
Na^{+}	660.0	12.5

[a]Enzyme activity was measured in phosphate buffer (pH 7.0) containing 1 mM Co^{++}.

Table 29. Enzyme activities on different proline peptides[a]

Proline peptides	Enzyme activity (mg Pro/ml supernatant/h)
L-Pro-Gly	1022.0
L-Pro-L-Ala	1220.0
L-Pro-L-Leu	1040.0
L-Pro-L-Phe	1412.0
Gly-L-Pro	40.0
L-Ala-L-Pro	596.0
L-Phe-L-Pro	534.0

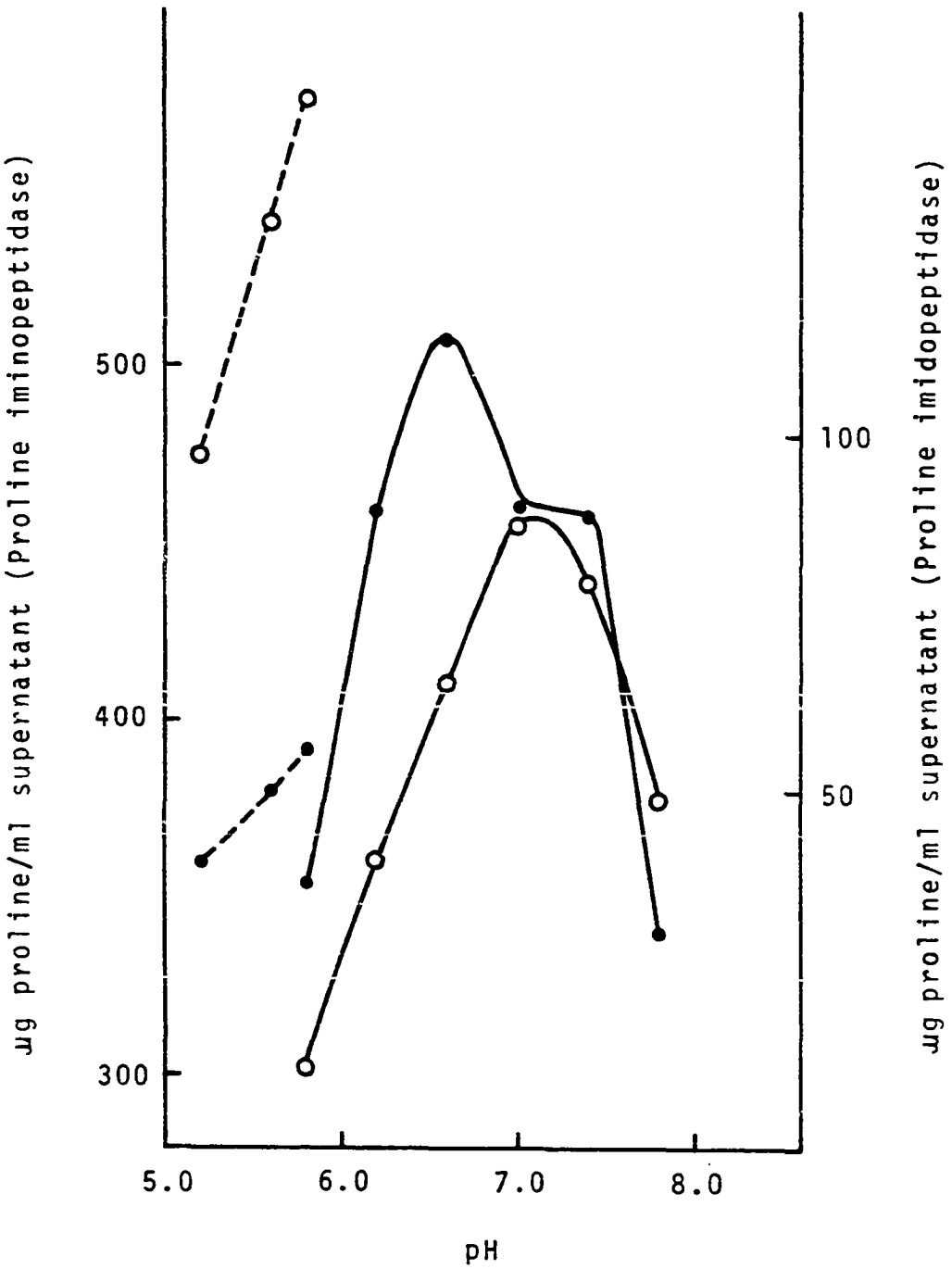
[a]Enzyme activity was measured in phosphate buffer (pH 7.0) containing 1 mM Co^{++}.

activated the hydrolysis of L-Pro-Gly, while Na^{+} had no effect. With Gly-L-Pro as substrate only Co^{++} showed an activating role; the other metals had no effect. No inhibitory influences were observed with any of the metal ions. The results indicated that two peptidases may be present; 1. A proline iminopeptidase activated by Co^{++} and Mn^{++}, and 2. A proline imidopeptidase activated by Co^{++}.

To further pursue this question, the optimum pHs for the enzyme activities were determined in phosphate and acetate buffer containing Co^{++}. The results are shown in Figure 34. Optimum activity for L-Pro-Gly was found to be pH 7.0, and for Gly-L-Pro, pH 6.6. Probably there is a Co^{++} and Mn^{++} activated proline iminopeptidase with optimum activity at pH 7.0, and a Co^{++} activated proline imidopeptidase with optimum activity at pH 6.6. Activity at lower pH values increased when phosphate buffer was exchanged with acetate buffer. Both enzymes showed activity at pH values found in Swiss cheese.

Side chains of peptides have often been found to influence enzyme activities (22, 23, 192, 246), so a limited study was performed to measure the influence of side chains on the enzyme activities of the proline peptidases. The results are shown in Table 29. The amino acid at the carboxyl

Figure 34. Influence of pH on proline iminopeptidase and imidopeptidase from P. shermanii, P-59, in phosphate and acetate buffers (0.05 M) at 32 C. (——), phosphate buffer, (----), acetate buffer. Symbols: o , proline iminopeptidase, ●, proline imidopeptidase


μg proline/ml supernatant (Proline iminopeptidase)
500
400
300
100
50
μg proline/ml supernatant (Proline imidopeptidase)
5.0
6.0
7.0
8.0
pH

end was found to influence the activity of proline imino-peptidase. With phenylalanine, an aromatic amino acid, the highest enzyme activity was measured. Alanine at the carboxyl end gave higher activities than glycine and leucine. The amino acid at the amino end had an even larger effect on the activity of proline imidopeptidase. Alanine or phenylalanine gave about 13 times higher activity with proline imido-peptidase than with glycine at the amino end. Proline iminopeptidase is extremely stable during storage at 3 C. No loss in activity was found after 30 days. With proline imidopeptidase, a loss in activity of 30% was found in the same time period. Enzyme activities for a few other sub-strates also were measured, as well as the effect of co-factors. The results are shown in Table 30. All peptides containing glycine at the amino end were activated by Mn^{++}. Co^{++} had a strong inhibitory effect on Gly-Gly and Gly-L-Leu. If the amino acid at the carboxyl end was aromatic, both Co^{++} and Mn^{++} activated the enzyme, with Mn^{++} as the most efficient activator. With L-Leu-Gly and L-Leu-L-Tyr, both Co^{++} and Mn^{++} inhibited the enzyme. With tripeptides, only low activities were observed, with Mn^{++} as an activator for Gly-Gly-Gly, and Co^{++} for L-Leu-Gly-Gly.

The results indicated that quite a few peptidases were present in the cell supernatant of P. shermanii, P-59, which probably would have some effect on the final composition of

Table 30. Effect of Co^{++} and Mn^{++} on enzyme activities on different dipeptides and tripeptides containing no proline in phosphate buffer (pH 7.0)

Peptides	Enzyme Activities (μMole/ml supernatant/h) Salts Added		
	None	Co^{++}	Mn^{++}
Gly-Gly	15.6	0.0	25.7
Gly-L-Leu	18.0	3.2	22.9
Gly-L-Tyr	2.8	10.1	14.2
Gly-L-Try	1.4	5.1	10.7
L-Leu-Gly	20.7	10.5	18.0
L-Leu-L-Tyr	30.3	16.9	24.0
Gly-Gly-Gly	0.0	0.0	2.5
L-Leu-Gly-Gly	0.0	1.8	0.0

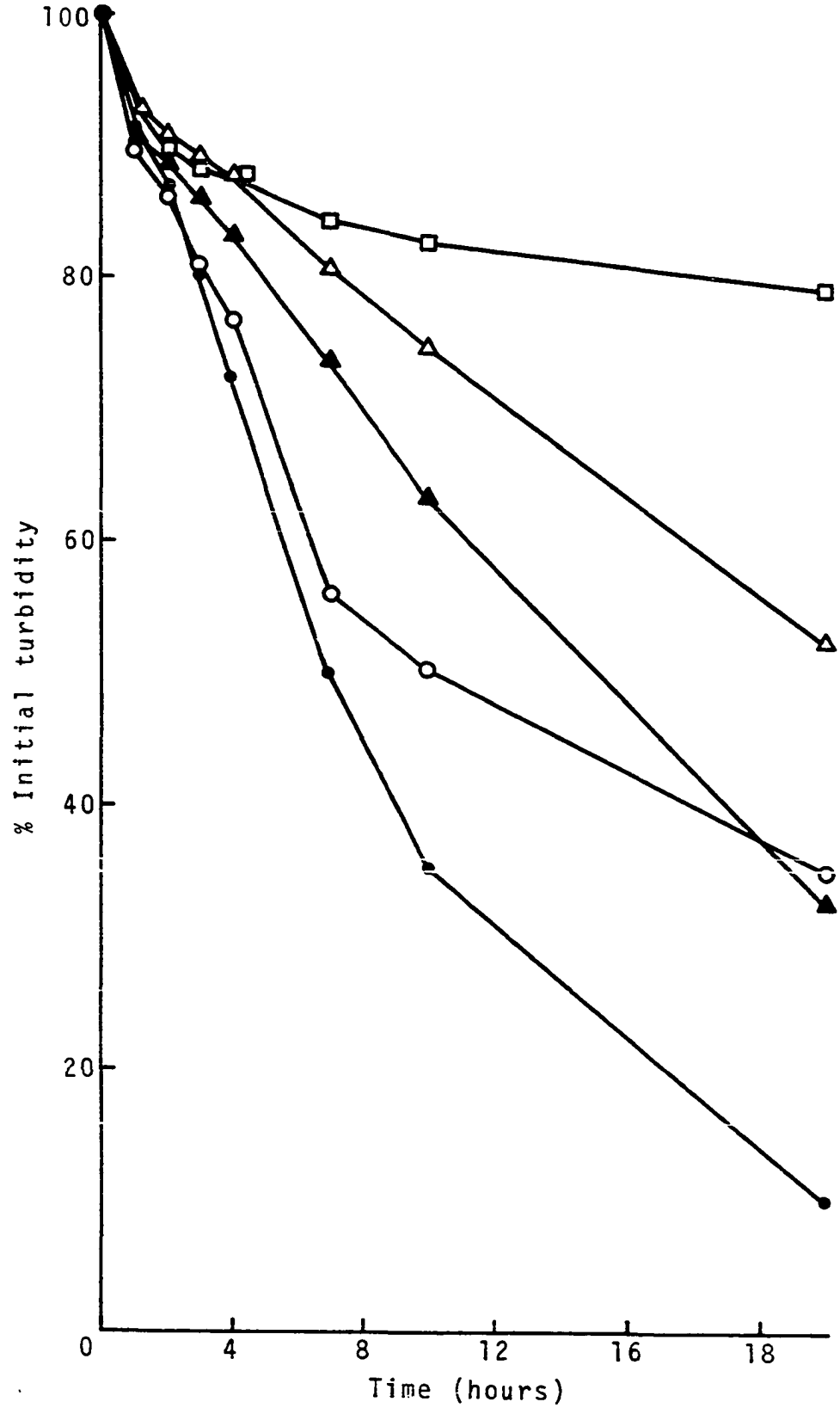
free amino acids in Swiss cheese. Specific peptidases are generally involved in hydrolysis of peptides containing proline (37); for example, Ryden (192) found two proline iminopeptidases and one proline imidopeptidase in a strain of Arthrobacter. Indications are that at least one of each of these enzymes are present in P. shermanii, P-59. To learn more about the numbers of peptidases present, more studies are needed. For the following experiments, the proline iminopeptidase was chosen because of its high activity.

Autolysis of propionibacteria in buffer systems

Effect of ionic strength A wide variety of bacterial species have been observed to be capable of autolysis. Autolysis has most generally been found to be most active while the cells are in the exponential growth phase, and the activity is generally very low in the stationary phase (33, 81, 208, 259). Higgins and Shockman (81) reported that the importance of autolysis is closely related to cell division, and, therefore, the activity decreases when growth decreases. However, if the autolytic system is important for releasing enzymes from propionibacteria, especially proline peptidases in this study, one would expect rapid autolysis in the late stationary phase or when cell numbers decrease, because that is the time when maximum proline production takes place.

The rate of autolysis has been shown to be influenced by ionic strength, usually as a sharp peak of activity over a narrow range (149). The autolytic activity of cells of P. shermanii, P-59, of different ages in phosphate buffers (pH 7.2) at ionic strengths from 0.01 to 1.25 was measured. Figure 35 shows autolysis measured as a function of time. The most rapid decrease in optical density was observed at an ionic strength of 0.3. The decrease in optical density was fairly linear up to 8 h after which the rate decreased. A slower decrease was observed at an ionic strength of 0.2.

Figure 35. Autolysis of 24-h-old cells of P. shermanii, P-59, in phosphate buffers (pH = 7.2) with different ionic strengths. Symbols: □ , I = 0.1, ○ , I = 0.2, ● , I = 0.3, △ , I = 0.75, ▲ , I = 1.25

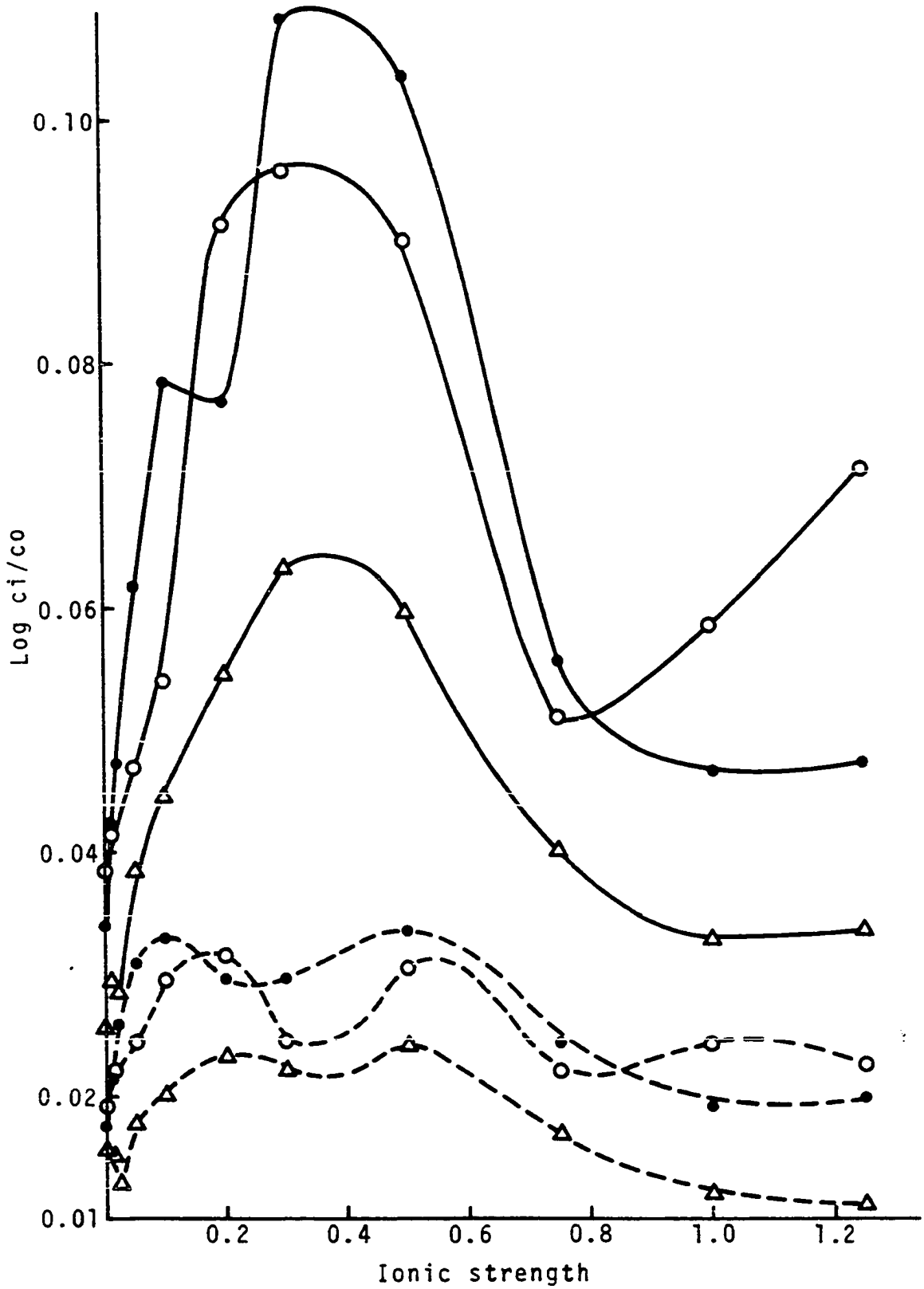

100
80
60
40
20
0
% Initial turbidity
4
8
12
16
18
Time (hours)

With ionic strengths higher and lower than 0.3 the rate of autolysis decreased.

Figure 35 show the results as they were directly measured with the spectrophotometer. However, the spectrophotometeric data were recalculated to log c_i/c_o where c_i is initial optical density and c_o is optical density at various times during incubation (259). This manipulation gave results which were easier to analyze. The results of autolysis of 24, 48, and 72-h-old cells after 1 and 4 h are shown in Figure 36. Autolysis was least in 48-h-old cells while the 24- and 72-h-old cells showed about the same degree of autolysis, with, perhaps, 72-h-old cells slightly faster. More than one peak was observed after 1 h. For 24-h-old cells, three peaks were found at ionic strengths of 0.2, 0.5, and about 1.0; for 48-h- and 72-h-old cells, two peaks, respectively, at 0.2 and 0.5, and 0.1 and 0.5. With increasing time, however, only one peak at about 0.3 was found for 24- and 48-h-old cells, with two peaks for 72-h-old cells at 0.1 and 0.3. The results indicated that more than one enzyme was involved in autolysis, and that ionic strength was influencing the optimum activities of the different enzymes. The literature (81) indicates from one autolytic enzyme in Streptococcus faecalis to three to four in Staphylococcus aureus and Bacillus subtilis.

By measuring autolysis at an ionic strength of 0.1 and

Figure 36. Autolysis of cells of P. shermanii, P-59, of different ages in phosphate buffers (pH = 7.2) as a function of ionic strengths. Autolysis is measured as log c_i/c_o where c_i is initial optical density and c_o is optical density at various times during incubation. (----) autolysis after 1 h, (——) autolysis after 4 h. Symbols: ○ , 24-h-old cells, △ , 48-h-old cells, and ● , 72-h-old cells


0.10
0.08
0.06
0.04
0.02
0.01
Log ci/co
0.2
0.4
0.6
0.8
1.0
1.2
Ionic strength

0.3 as a function of time (Figure 37), one sees that the rate of autolysis for a 24-h-old culture at an ionic strength of 0.3 started to increase after 5 h in comparison with a 72-h-old culture. At an ionic strength of 0.1, the rate of autolysis for a 72-h-old culture of P. shermanii, P-59, was much faster than for a 24- and 48-h-old culture. The autolytic activity for a 24-h-old culture decreased with time. This was an indication that the different autolytic enzymes involved in autolysis, have different stabilities. Autolytic enzymes are generally very unstable. One natural assumption is that the autolytic system is heavily regulated during the exponential growth phase (81). When the growth rate decreases this may lead to less autolytic activity as shown by 48-h-old cells. However, with age, the regulatory system may be inactivated earlier than the enzymatic system, which will explain the increase in autolysis of 72-h-old cells.

Effect of pH on autolysis Seventy-two-h-old cells of P. shermanii, P-59, were used in this experiment. Phosphate and acetate buffers at an ionic strength of 0.3 were employed and the results are shown in Figure 38. One optimum peak was found at pH 7.2, another at pH 6.0, and there was an indication of a third peak at pH 5.2. The autolytic activities of the cells were less in acetate buffer than in

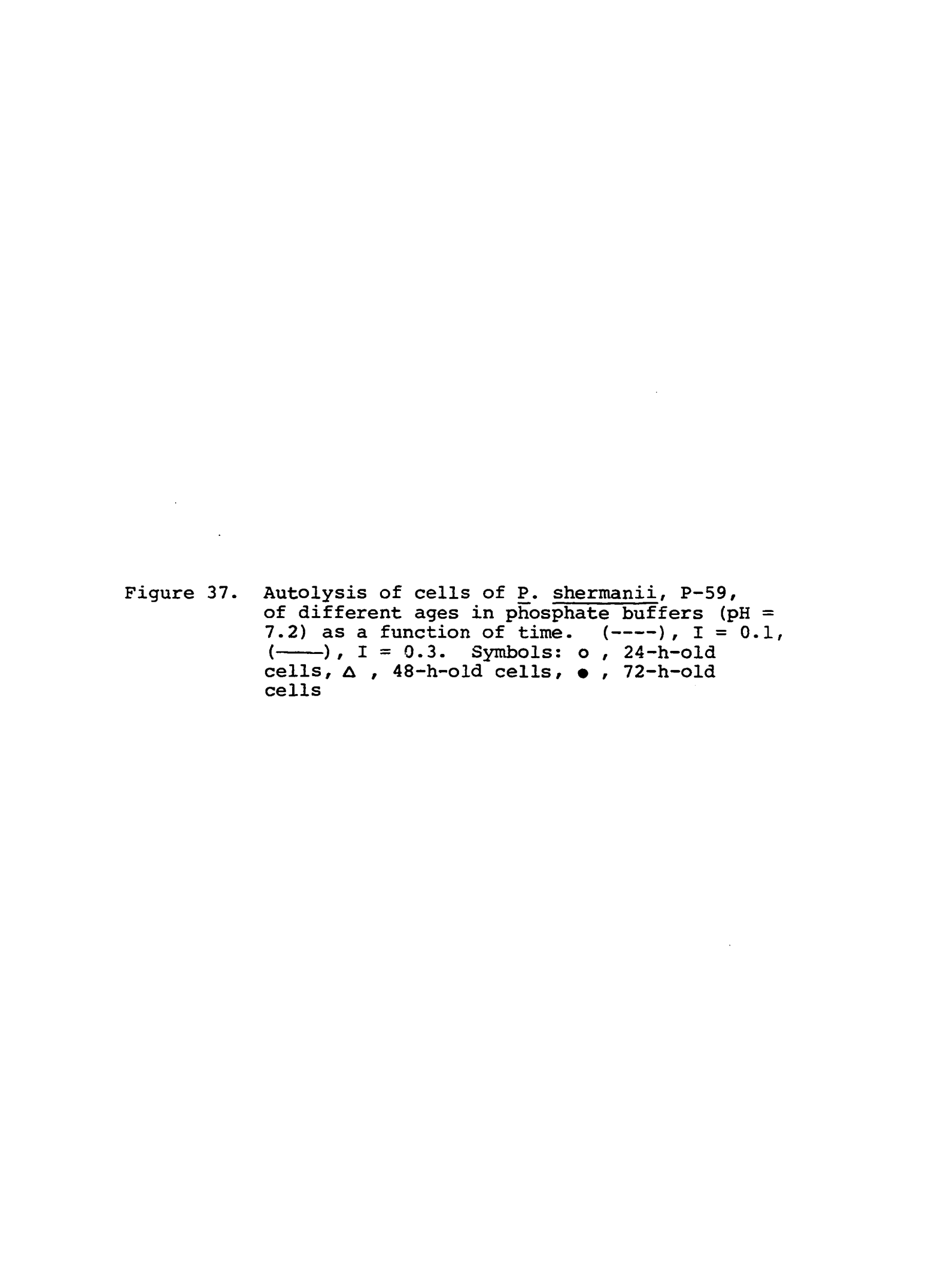


Figure 37. Autolysis of cells of P. shermanii, P-59, of different ages in phosphate buffers (pH = 7.2) as a function of time. (----), I = 0.1, (———), I = 0.3. Symbols: o , 24-h-old cells, Δ , 48-h-old cells, ● , 72-h-old cells

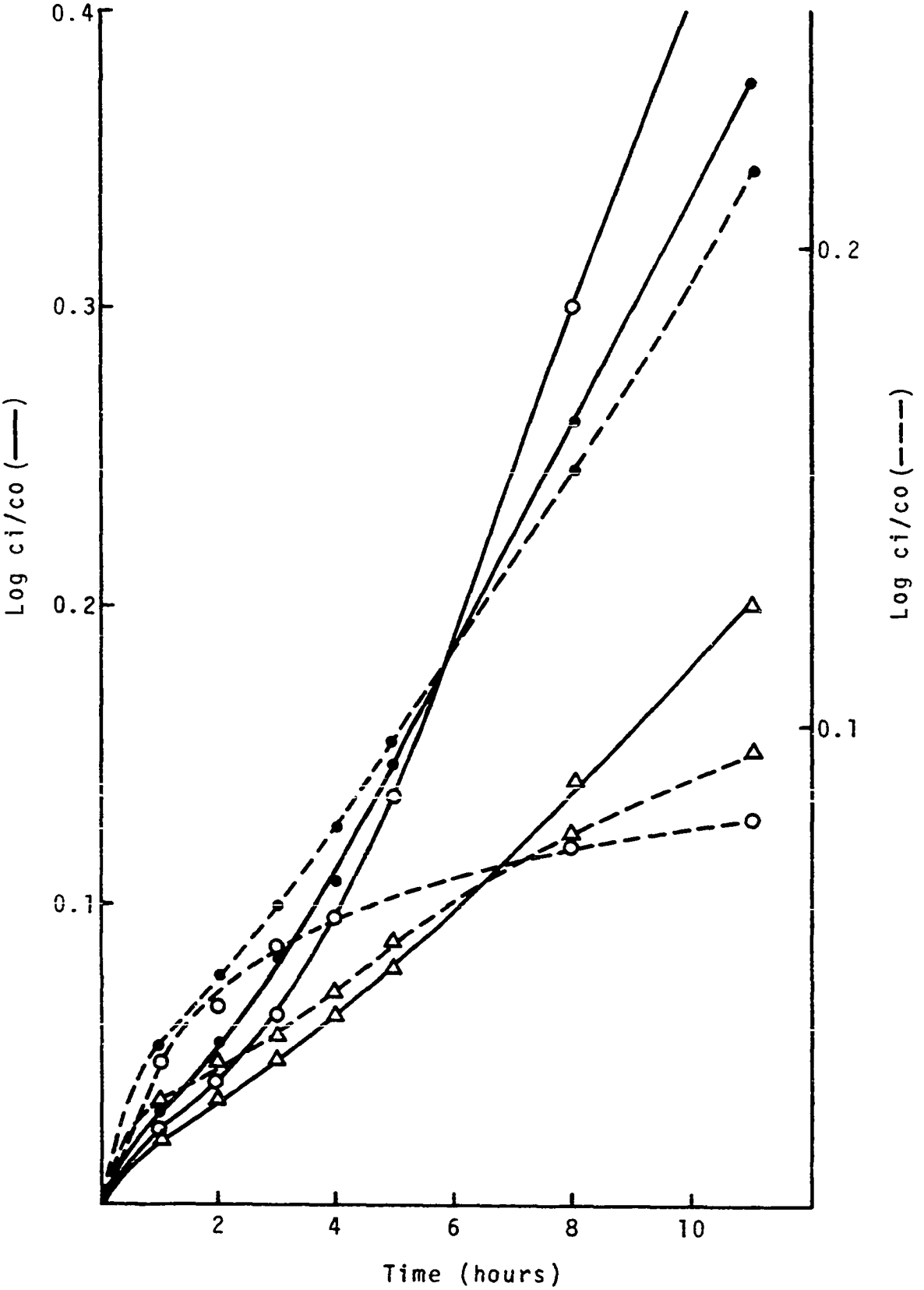
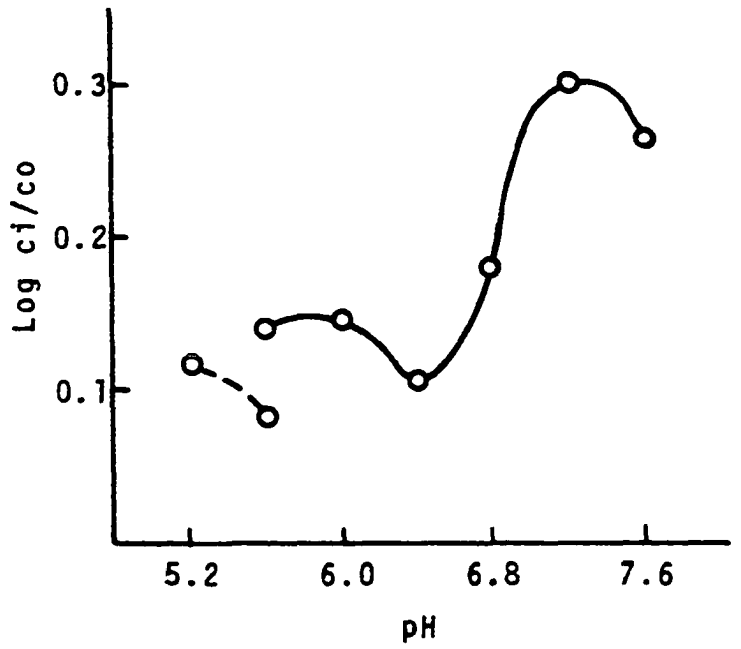
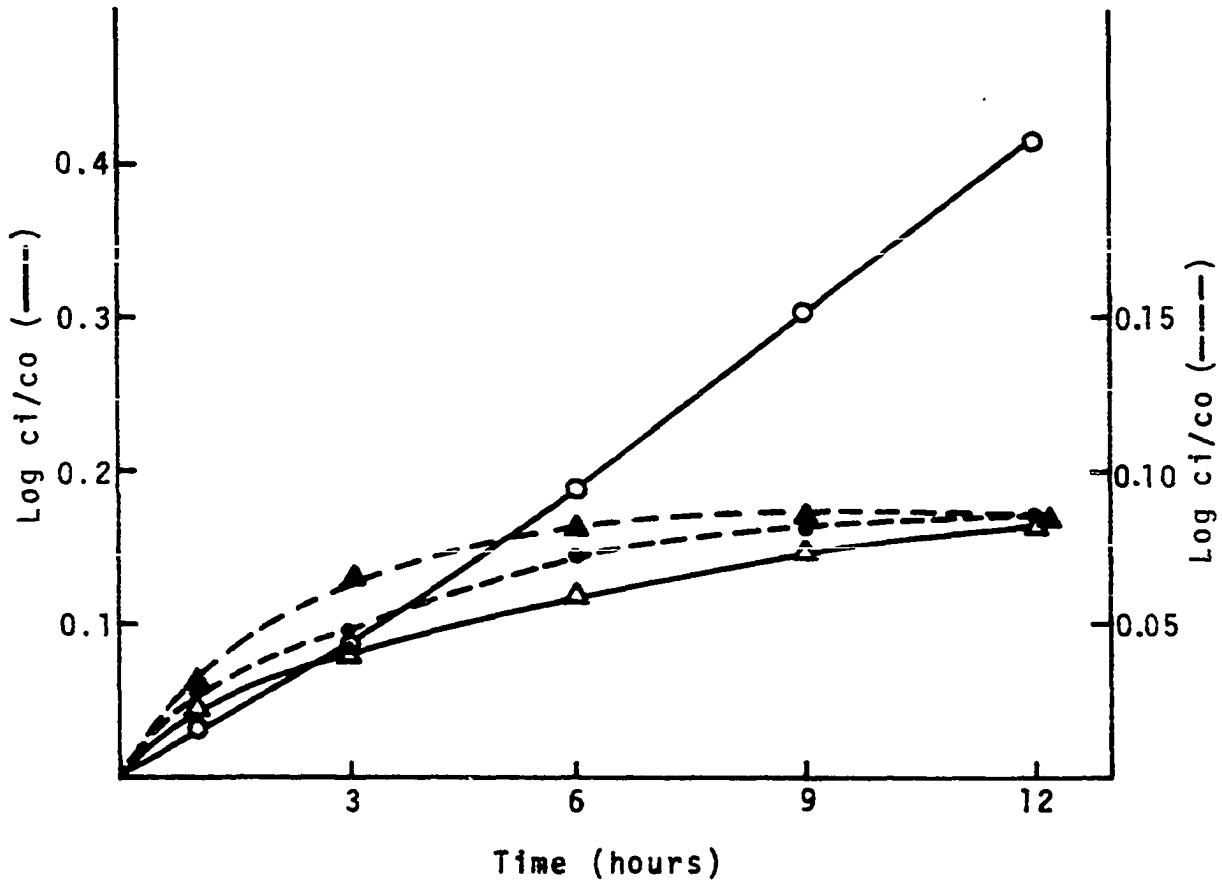

0.4
0.3
0.2
0.1
Log ci/co (——)
0.2
0.1
Log ci/co (– – –)
2
4
6
8
10
Time (hours)

Figure 38. Influence of pH on autolysis of 72-h-old cells of P. shermanii, P-59, in phosphate and acetate buffers (I = 0.3)

Figure 39. Influence of pH on rate of autolysis of 72-h-old cells of P. shermanii, P-59. Symbols: o , Phosphate buffer, (pH = 7.2, I = 0.3), Δ , Phosphate buffer (pH = 7.2, I = 0.3), ● , Acetate buffer (pH = 7.2, I = 0.3), ▲ , Milk salt solution (pH = 5.6, I = 0.3)

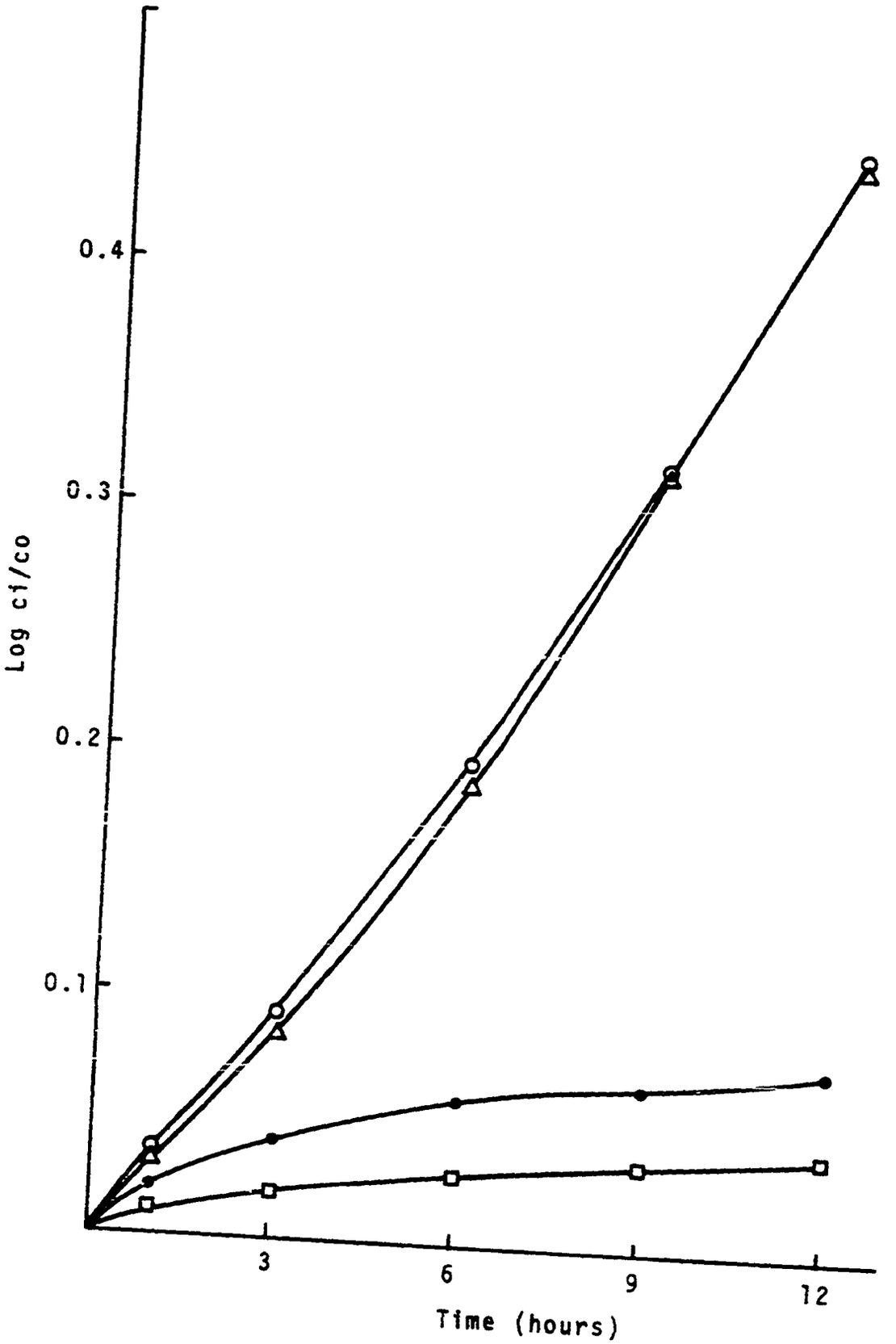

0.3
0.2
0.1
Log ct/co
5.2
6.0
6.8
7.6
pH


0.4
0.3
0.2
0.1
Log ct/co (——)
0.15
0.10
0.05
Log ct/co (- - -)
3
6
9
12
Time (hours)

phosphate buffer. The results, of course, indicate again that more than one enzyme is involved. From information in the literature, N-acetylglucosaminidase, bridge-splitting peptidases, and N-acetylmuramyl-L-alanine amidases have been involved, which show pH-activities closely related to the different peaks observed (81, 149, 259), but far more work must be done to determine which autolytic enzymes are involved.

Figure 39 shows the rate of autolysis at different pH values. As also seen from Figure 38, the fastest autolysis took place at pH 7.2 and the change was linear. Both phosphate and acetate buffer at pH 5.6 showed decreasing rates with time indicating inactivation. The inactivation may not necessarily take place in a complex surrounding as in cheese or in a medium, because protective compounds may be present. Also included in the figure is an artificial salt solution which is closely related to the salts in a milk serum at pH 5.6. The behavior was as for acetate buffer. These results indicated that autolysis of P. shermanii, P-59, will take place at pH values and ionic strengths found in Swiss cheese.

Figure 40. Autolysis of different strains of propionibacteria in phosphate buffer (pH = 7.2, I = 0.3). Symbols: ○ , P. shermanii, P-59, △ , P. shermanii, P-24, ● , P. freudenreichii, P-19, P. shermanii, P-1, P. zeae, P-35, □ , P. pentosaceum, P-9


0.4
0.3
0.2
0.1
Log c1/co
3
6
9
12
Time (hours)

Variation in autolysis between different strains of propionibacteria In an earlier experiment, it was shown that different strains of propionibacteria produced proline at different rates. This experiment was done to see if differences in autolysis by different strains of propionibacteria, could be related to proline production. The results are shown in Figure 40. P. shermanii, P-24 and P-59, autolyzed faster than any of the other strains, and these strains also were the most rapid proline producers. P. shermanii, P-1, P. freudenreichii, P-19, and P. zeae, P-35, showed the same degree of autolysis. Of these bacteria, P. freudenreichii, P-19, produced proline fastest, followed by P. shermanii, P-1, and P. zeae, P-35, so no correlation between proline production and autolysis was found between these strains. P. pentosaceum, P-9, autolyzed slowest and also was the strain that produced proline slowest. P. intermedium, P-74, also was included in this study, but autolysis was not observed, which also fitted the proline production, for no proline was produced after 15 days. The experiment showed large differences in autolysis between fast, intermediate, and slow proline producers.

Autolysis of propionibacteria in media during active growth

Influence of different media on growth and autolysis of P. shermanii, P-59 In earlier experiments, it was found that proline production was faster in SLB than in SLB in which yeast extract had been exchanged with a defined vitamin and salt solution. Therefore, these media were used in this experiment. Also included were two media with a defined vitamin solution containing, respectively, 0.5 and 2% Casamino acids. Growth was measured by total cells numbers and optical density. The results are shown in Figures 41 and 42.

The fastest growth took place in SLB, and the growth rate was somewhat slower in the other media. Maximum cell numbers were reached after 2 days in SLB, after 3 days in SLB without yeast extract and in the medium containing 0.5% Casamino acids, and after 4 days in the final medium. Maximum optical density was found after 3 days for all four media, which then started to decrease. The optical density or cell mass was much higher for P. shermanii, P-59, grown in SLB than in the same medium without yeast extract. By increasing the content of Casamino acids, the optical density also increased. To better compare the differences in autolysis, the optical density readings were transformed to log c_i/c_o, and the results are shown in Figure 42. The fastest degree of autolysis was observed in SLB, then followed by SLB without

Figure 41. Influence of different media on growth and autolysis of P. shermanii, P-59. (———), optical density, (----), cell numbers. Symbols: o, SLB, Δ, SLB in which yeast extract has been exchanged with a defined vitamin solution, ◘, defined medium containing 0.5% Casamino acids, ◒, defined medium containing 2% Casamino acids

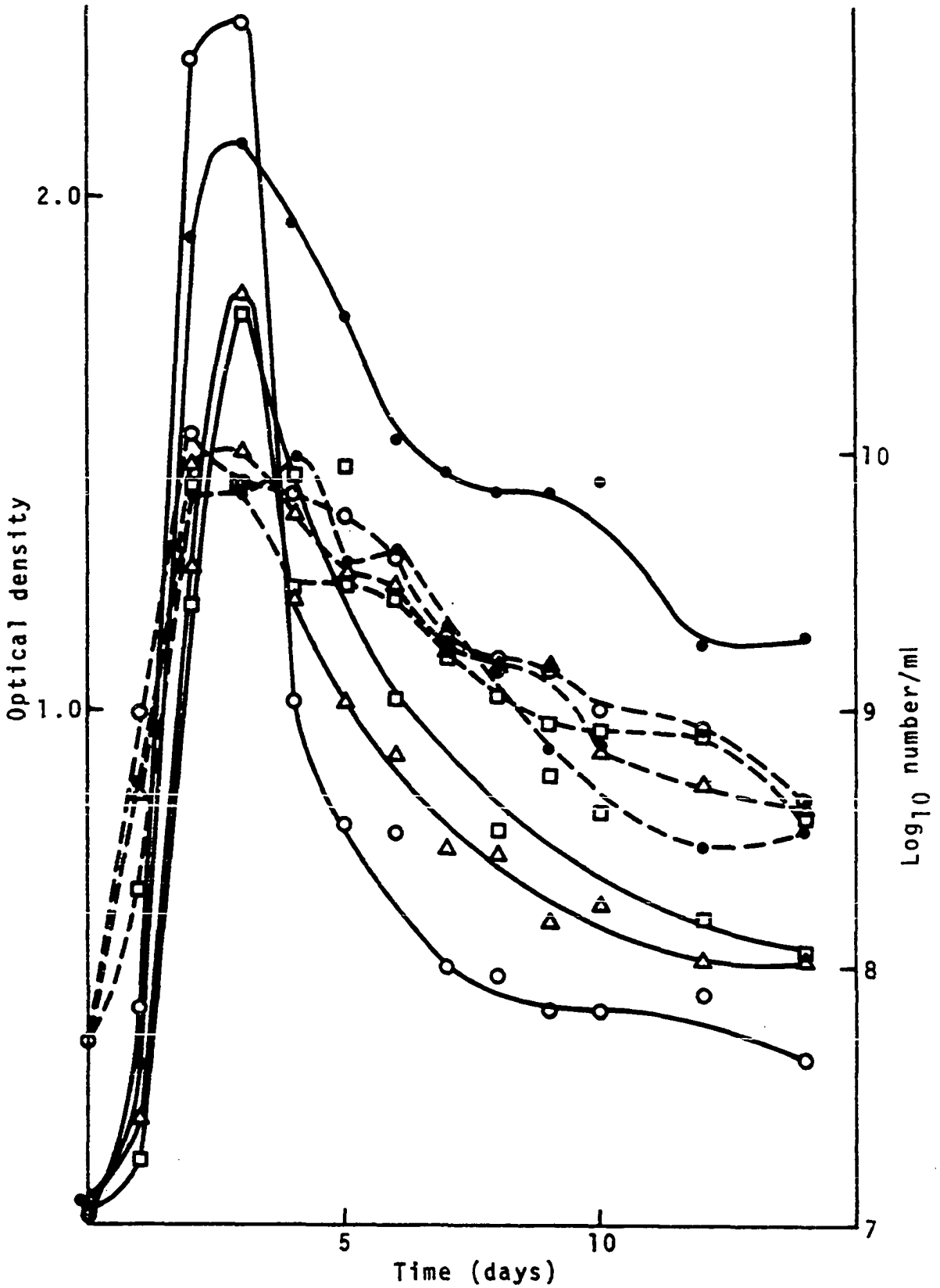
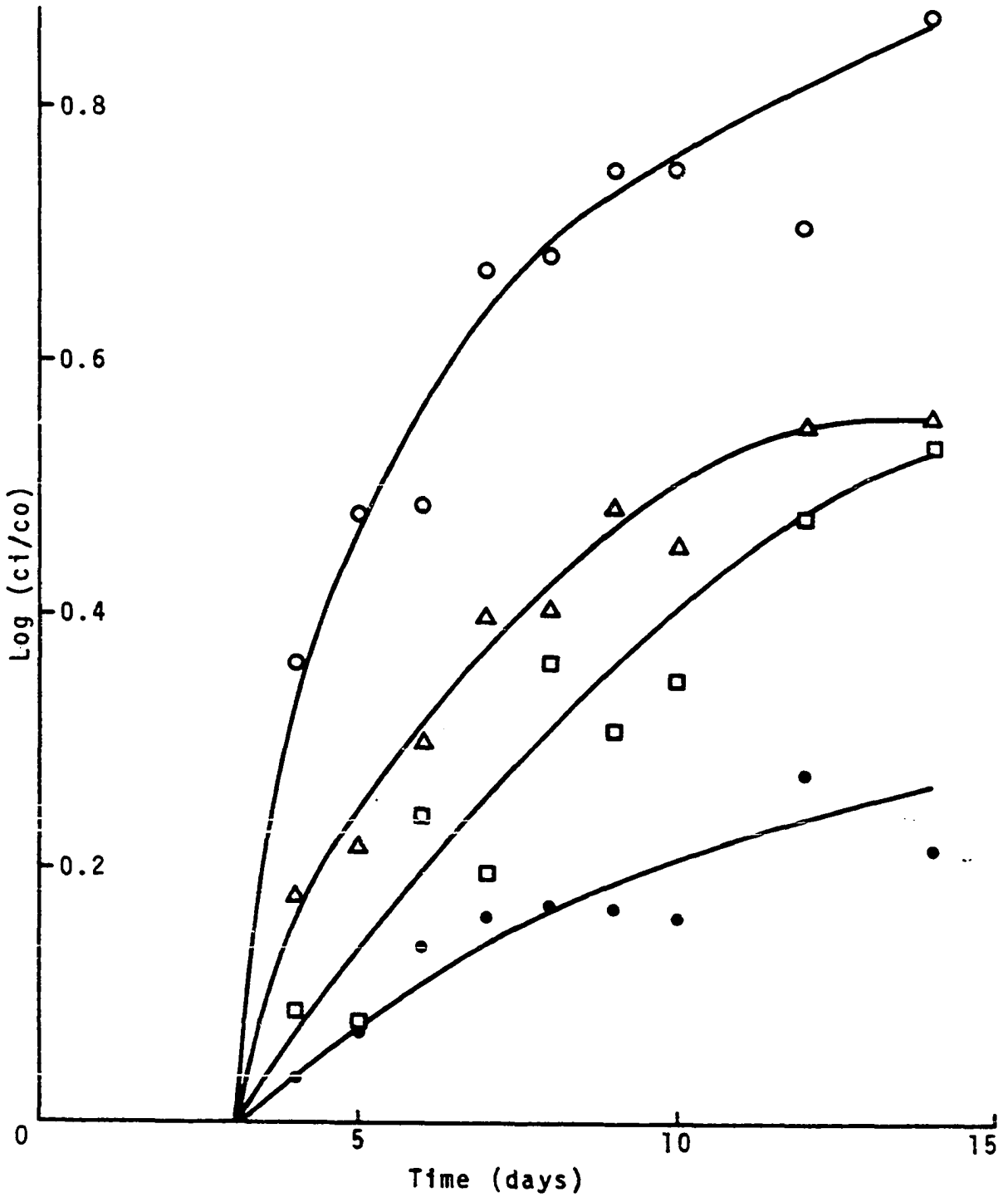

Optical density
2.0
1.0
5
10
Time (days)
10
9
8
7
Log10 number/ml

Figure 42. Autolysis of P. shermanii, P-59, in different media as measured by log c_i/c_o where c_i is initial optical density and c_o is optical density at various times during incubation. Symbols: o , SLB, Δ , SLB in which yeast extract has been exchanged with a defined vitamin solution, □ , defined medium containing 0.5% Casamino acids, ● , defined medium containing 2% Casamino acids


0.8
0.6
0.4
0.2
0
Log (ct/co)
5
10
15
Time (days)

yeast extract. The differences in autolysis will explain the difference in rate of proline production, which was shown in an earlier experiment (Figure 7). The slowest autolysis was found in the medium containing 2% Casamino acids.

The addition of a defined vitamin solution containing high amounts of salts instead of yeast extract, decreased the autolysis rate, probably due to changes in ionic strength. The loss of an activating substance in yeast extract may be another, but less probable, possibility. By exchanging Trypticase with 0.5% Casamino acids another decrease was seen, which probably was caused by the higher amounts of free amino acids in the medium. By further increasing the free amino acid content, a continuous decrease in autolysis took place. This shows that medium composition will affect autolysis. The microenvironment in the cheese will influence the autolytic activity of propionibacteria, but the high content of peptides in the cheese, suggests that fairly rapid autolysis will take place. However, the effect of proteins is unknown.

Strain differences The same strains used to determine differences in autolysis in phosphate buffers, were used in this experiment. P. shermanii, P-59, and P. pentosaceum, P-9, were especially selected to follow both cell

numbers and optical density. The results are shown in Figure 43. Maximum optical density for P. shermanii, P-59, was reached after 2 days, and the stationary phase when cell numbers were measured also was reached after 2 days, but cell numbers still increased slightly up to the 3rd day. During the same time period, the optical density decreased rapidly. This indicated that autolysis had started before optimum cell numbers were reached, probably slightly before the 2nd day.

When P. shermanii, P-59, was compared with P. pentosaceum, P-9, it is evident that the growth rate measured by optical density is the same, although measurement of total cell numbers showed slower growth rates for P. pentosaceum, P-9. The decrease in optical density of the P. pentosaceum strain was slower than for P. shermanii, P-59.

Figure 44 shows the difference in autolysis between all seven strains. The differences in autolysis among the four strains with the highest degree of autolysis, followed their rate of proline production as shown in Figure 23 and 26 for those strains. Of the remaining three strains, P. zeae, P-35, produced proline somewhat faster and it's autolysis started earlier, followed by P. pentosaceum, P-9, and P. intermedium, P-74, which was the slowest proline producer. A definite correlation was found between proline production and autolysis.

Figure 43. Growth and autolysis of P. shermanii, P-59, and P. pentosaceum, P-9, in SLB. (———), optical density, (----), cell numbers. Symbols: o , P. shermanii, P-59, ● , P. pentosaceum, P-9

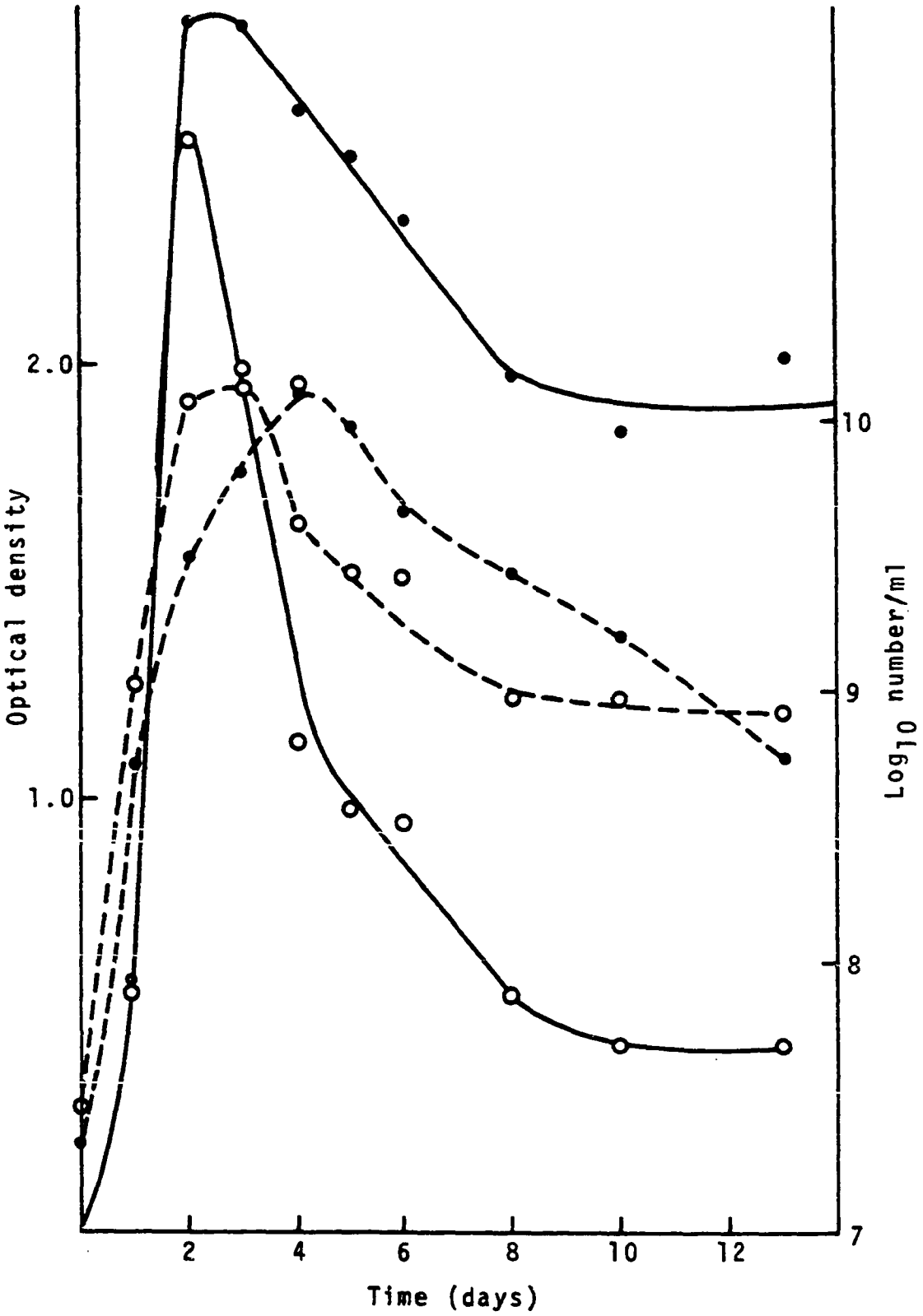
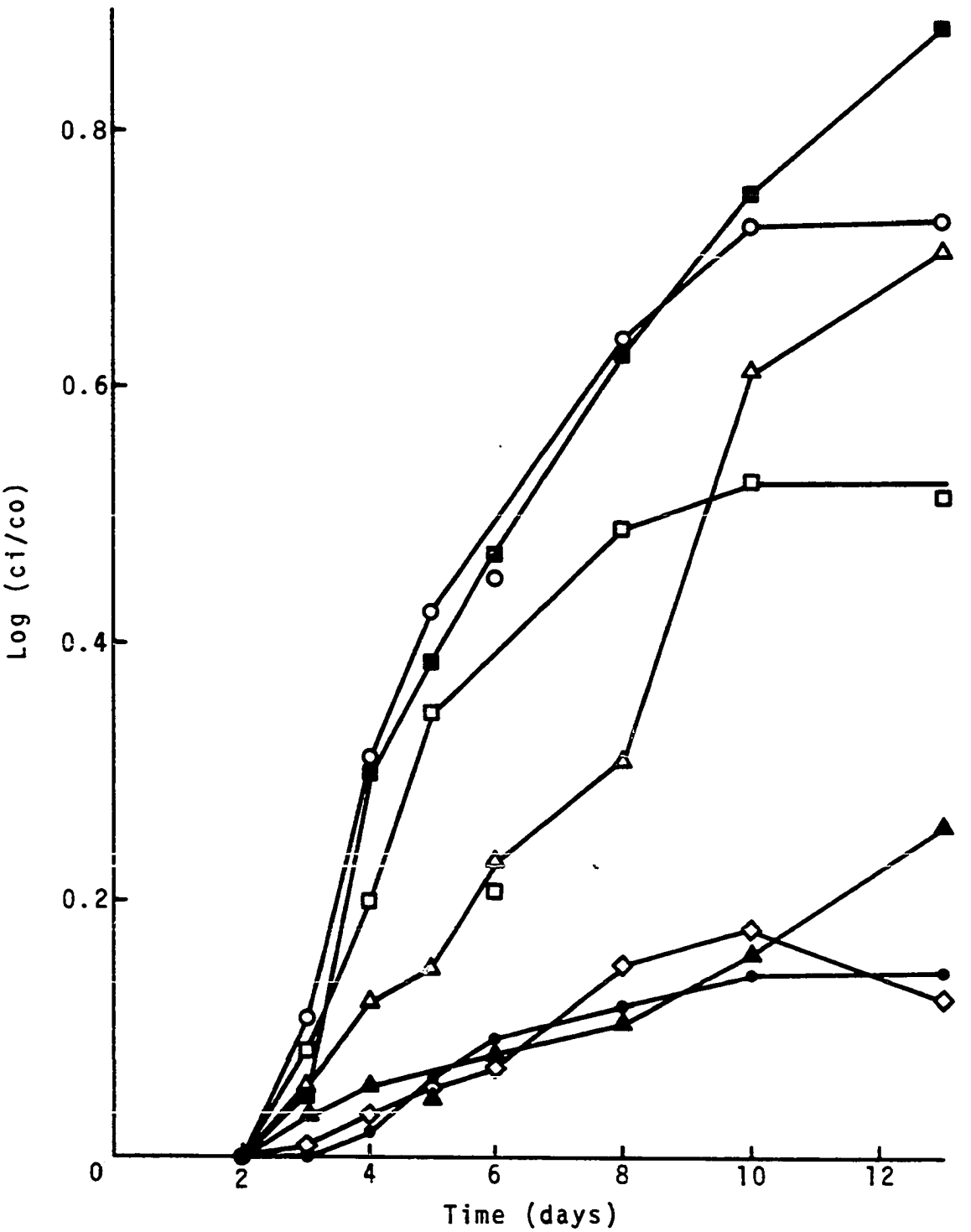

Optical density
2.0
1.0
Log10 number/ml
10
9
8
7
2
4
6
8
10
12
Time (days)

Figure 44. Autolysis of seven strains of propionibacteria in SLB as measured by log c_i/c_o. Symbols: o , P. shermanii, P-59, □ , P. freudenreichii, P-19, ■ , P. shermanii, P-24, Δ , P. shermanii, P-1, ▲ , P. zeae, P-35, ◇ , P. pentosaceum, P-9, ● , P. intermedium, P-74


0.8
0.6
0.4
0.2
0
Log (ci/co)
2
4
6
8
10
12
Time (days)

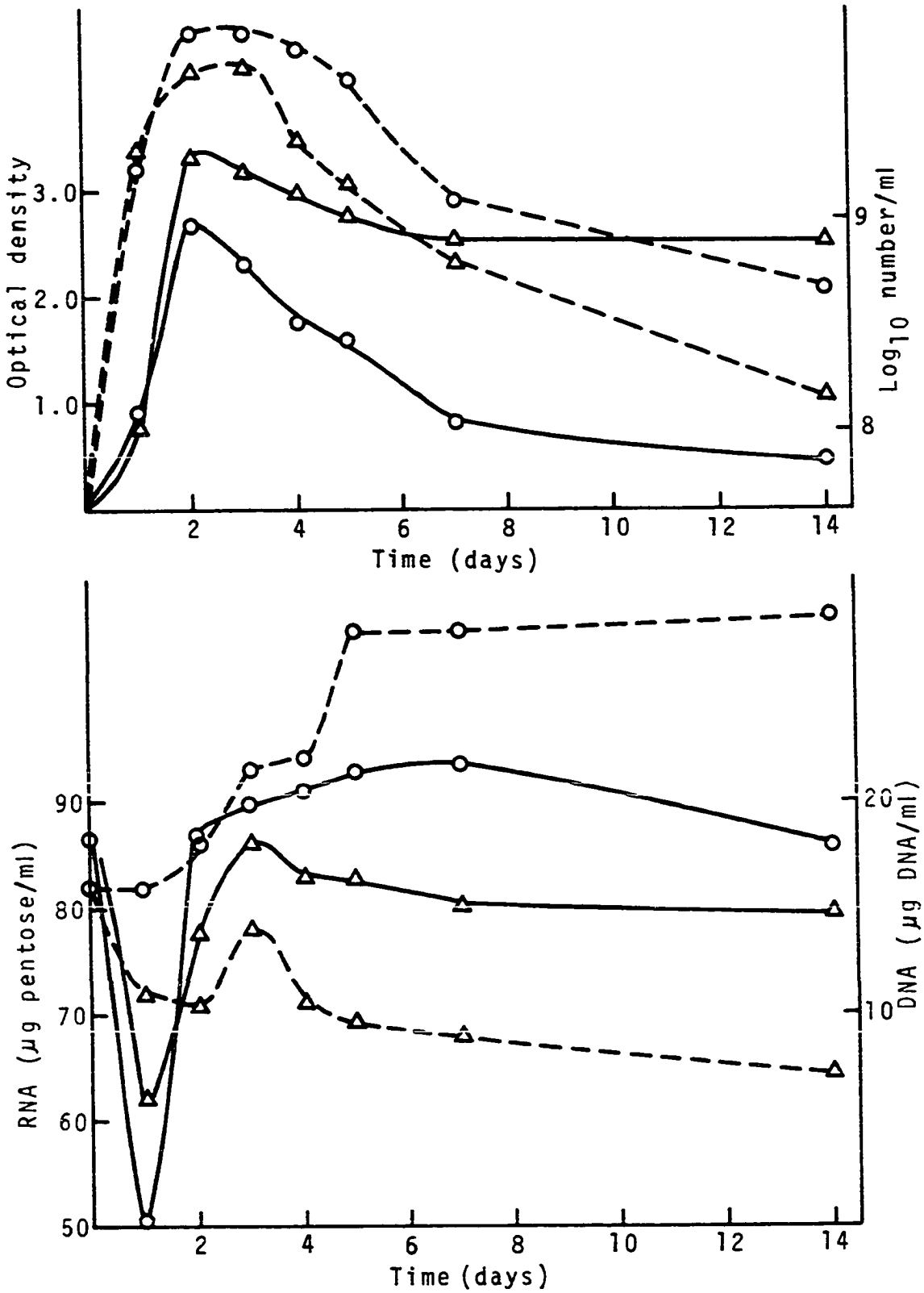
Proline production in relation to autolysis

Cell autolysis and proline production appear to follow each other. To pursue this supposition further, it was decided to follow release of RNA, DNA, and proline iminopeptidase in relation to cell autolysis. P. shermanii, P-59, a fast proline-producer, and P. pentosaceum, P-9, a slow proline-producer were used.

Figure 45 depicts their growth measured by optical density and total cell numbers, and shows the same results as for the last experiment. Figure 46 shows the RNA and the DNA in the cell-free medium during growth and autolysis of their cells. RNA release was followed using the orcinol reaction which combines with ribose, although other pentoses will interfere with the reaction. Since yeast extract contains both ribo- and deoxyribonucleotides, a high control value, expected for the medium, was observed. A rapid decrease in RNA occurred with both species during the first day, most noticeably for P. shermanii, P-59, which usually grows faster than P. pentosaceum, P-9. A rapid release of RNA took place between the first and the 2nd day. Undoubtedly, between the first and the 2nd day, conditions became adverse. During this time, breakdown of RNA usually occurs inside the cell, and RNA fragments are released into the medium. This was earlier observed for starved S. lactis

Figure 45. Changes in optical density and total cell numbers during growth of P. shermanii, P-59, and P. pentosaceum, P-9, in SLB at 32 C. (——), optical density, (----), cell numbers. Symbols: o , P. shermanii, P-59, Δ , P. pentosaceum, P-9

Figure 46. Changes in RNA and DNA released into medium during growth of P. shermanii, P-59, and P. pentosaceum, in SLB at 32 C. (——), RNA, (----), DNA. Symbols: o , P. shermanii, P-59, Δ , P. pentosaceum, P-9


Optical density
3.0
2.0
1.0
Log10 number/ml
9
8
2
4
6
8
10
12
14
Time (days)
RNA (µg pentose/ml)
90
80
70
60
50
DNA (µg DNA/ml)
20
10
2
4
6
8
10
12
14
Time (days)

(232), and this loss took place before any loss of viability. The RNA content of the medium with P. shermanii, P-59, continued to increase slowly up to the 7th day, while from P. pentosaceum, P-9, less RNA was released and the maximum content was reached at the 3rd day.

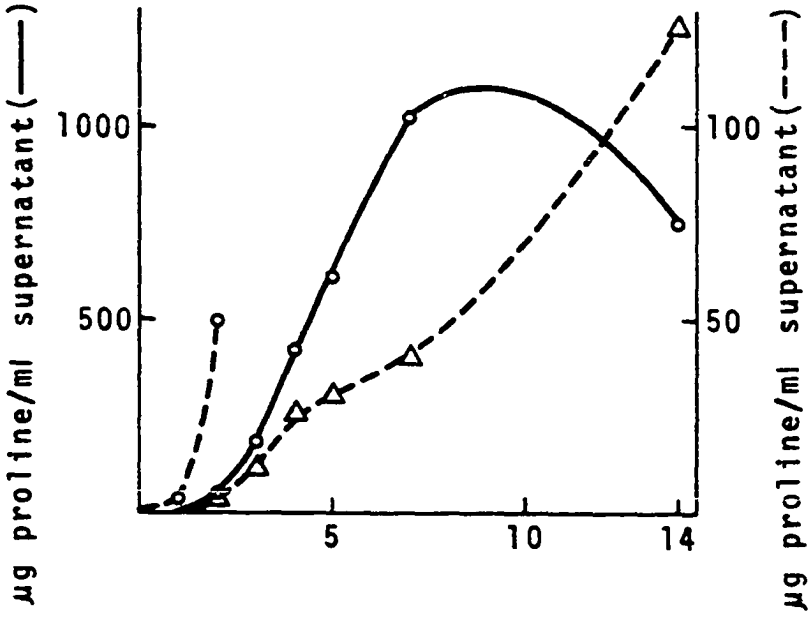
Burton's method (24), which was used for DNA analysis, is a much more specific reaction with deoxyribose, than is orcinol with ribose. For P. shermanii, P-59, the amount of deoxyribose measured was constant the first day after which it started to increase continuously up to the 5th day after which it was constant. However, DNA from P. pentosaceum, P-9, decreased the first day, and then started to increase after the 2nd day. After the 3rd day, DNA started to again decrease. Thomas et al. (233) reported that DNA was released from S. lactis as a macromolecule. The reason for the decrease in RNA and DNA after they have reached a maximum value, was probably caused by the fact that the experiment was performed in a dynamic system where even when cell numbers are decreasing, new cells are being formed. Also, when the cells autolyze, RNases and DNases and other nucleotide degrading enzymes are probably released. DNA release started before any decrease in optical density was observed which indicates that autolysis started even when a rapid increase in cell numbers was occurring.

The release of proline iminopeptidase into the medium is

shown in Figure 47. After 1 day, a low proline iminopeptidase activity was observed in the cell-free medium with P. shermanii, P-59, which increased slowly up to the 2nd day. After 2 days, the amount of enzyme released increased faster, and the increase was linear from the 3rd to the 7th day after which an optimum content was reached. The amount of proline peptidase then decreased, possibly either by inactivation or by degradation by intracellular proteinase. For P. pentosaceum, P-9, no activity was observed before 2 days, after which a continuous increase took place over the next 14 days. However, it can be remarked that the enzyme activity was about 10 times higher when the maximum amount of enzyme had been released from P. shermanii, P-59, than from P. pentosaceum, P-9.

The release of enzyme from P. shermanii, P-59, started before any decrease in optical density had begun and also before any measurable DNA had been released. This indicated that some autolysis had taken place during the logarithmic growth phase. Another possibility may be that the enzyme is an extracellular enzyme. But in contrast to the last assumption was the fact that most of the enzyme was released during the time when the maximum decrease in optical density was taking place. The release of proline iminopeptidase from P. pentosaceum, P-9, was much slower. It

Figure 47. Proline production by proline iminopeptidase released into the medium during growth of P. shermanii, P-59, and P. pentosaceum, P-9, in SLB at 32 C. Symbols: ○ , P. shermanii, P-59, Δ , P. pentosaceum, P-9


µg proline/ml supernatant(——)
1000
500
5
10
14
100
50
µg proline/ml supernatant(---)

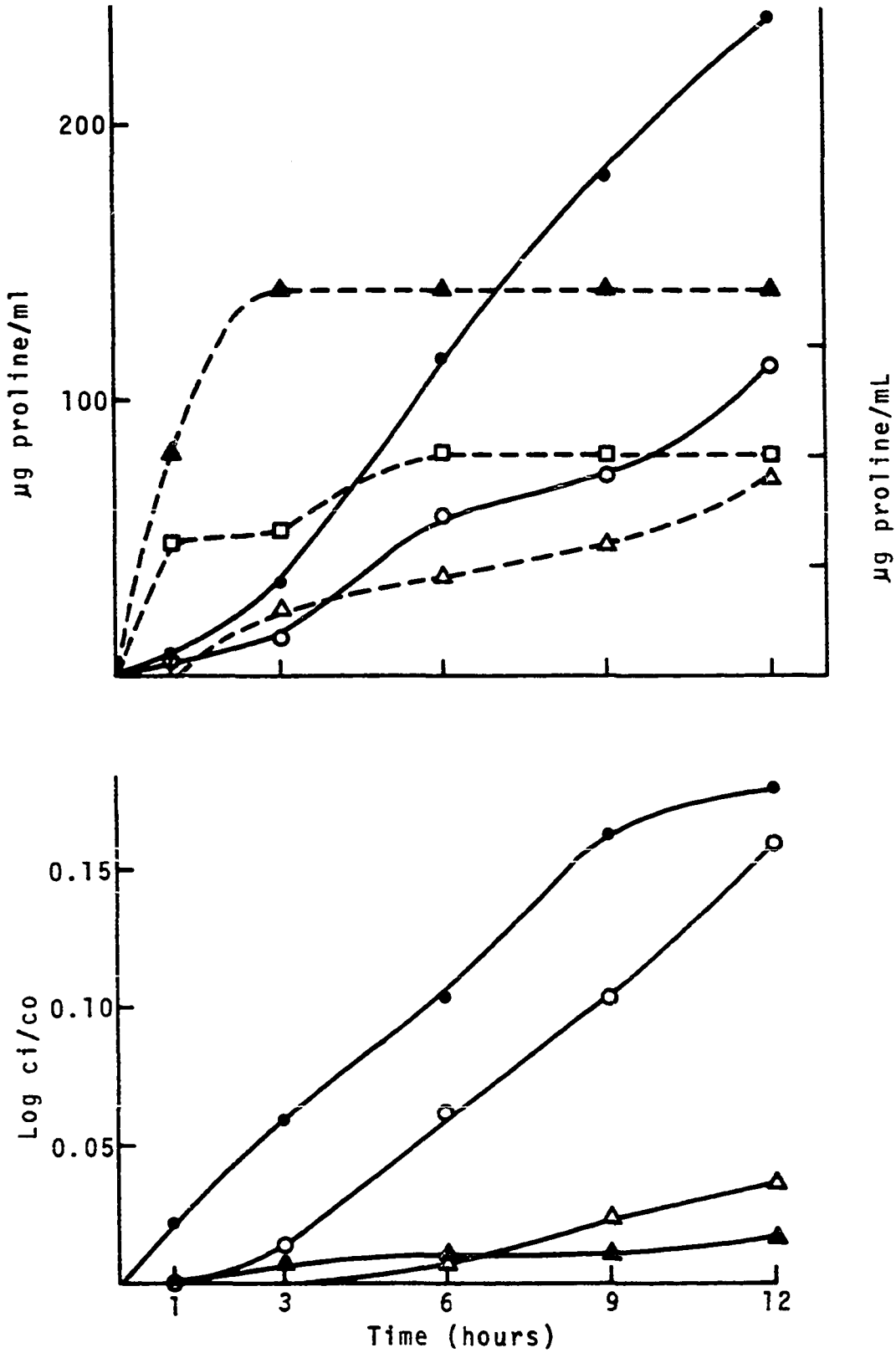
started later, but, again, before any decrease in optical density occurred. The release of the enzyme was much slower paralleling the slow decrease in optical density.

To decide if the proline iminopeptidase was extracellular, washed cells of P. shermanii, P-59, and P. pentosaceum, P-9, were suspended in phosphate buffer, (pH 7.0, and ionic strength 0.3) at high optical densities. Autolysis was measured by diluting the samples in either phosphate buffer or water, and enzyme activities were measured in phosphate buffer after removal of cells. The results are shown in Figures 48 and 49. Autolysis was already measurable after 1 h when the cells of P. shermanii, P-59, were diluted in water. Over the entire time period, larger values were observed for the autolysis carried out in water than in phosphate buffer. This indicated that the cells were weakened before they were diluted in water, and that the change in osmotic pressure increased the autolysis. There is a possibility that holes may be formed in the cell which may leak the enzyme. For P. pentosaceum, P-9, no large differences were observed between dilution in water and buffer, and the degree of autolysis was very low.

Figure 48 shows that the leakage of proline took place with both species, probably more before 2 h. More intracellular proline was observed with P. shermanii, P-59, than

Figure 48. Proline production from proline iminopeptidase released during autolysis in phosphate buffer (pH = 7.0, I = 0.3) of 72-h-old cells from P. shermanii, P-59, and P. pentosaceum, P-9. Symbols: ● , Proline iminopeptidase activity from P. shermanii, P-59, after 6 h assay, o , Proline iminopeptidase activity from P. shermanii, P-59, after 3 h assay, Δ , Proline iminopeptidase activity from P. pentosaceum, P-9, after 6 h assay, ▲ , Release of intracellular proline from P. shermanii, P-59, □ , Release of intracellular proline from P. pentosaceum, P-9

Figure 49. Autolysis of P. shermanii, P-59, and P. pentosaceum, P-9, in phosphate buffer (pH = 7.0, I = 0.3) by dilution in phosphate buffer (pH = 7.0, I = 0.3) or water. Symbols: ● , P. shermanii, P-59, in water, o , P. shermanii, P-59, in phosphate buffer, ▲ , P. pentosaceum, P-9, in water, Δ , P. pentosaceum, P-9, in phosphate buffer


200
100
µg proline/ml
µg proline/mL
0.15
0.10
0.05
Log ci/co
1
3
6
9
12
Time (hours)

with P. pentosaceum, P-9. No enzyme activity was observed with any of the species at zero time, but the activity for proline iminopeptidase increased rapidly for P. shermanii, P-59, which autolyzed fastest. Only small activities were observed for P. pentosaceum, P-9, which showed a low degree of autolysis. The results further verified the fact that the proline iminopeptidase is an intracellular enzyme, because its presence increased with autolysis.

Conclusion

Four or five peptidases were found in P. shermanii, P-59, of which one was a proline iminopeptidase activated by Co^{++} and Mn^{++} and another a proline imidopeptidase activated by Co^{++}. Both enzymes were active at pH values found in Swiss cheese.

Rapid autolysis took place in buffer systems with P. shermanii, P-59. More than one enzyme was observed in this process. Autolytic activities were found at ionic strengths close to those in cheese, and at pH values found in cheese. The degree of autolysis by different strains of propionibacteria was correlated with their proline-producing capacity.

Release of RNA, DNA, and proline iminopeptidase by P. shermanii, P-59, and P. pentosaceum, P-9, was followed in SLB. The release of proline iminopeptidase paralleled the rate of autolysis. The proline iminopeptidase was an intracellular

enzyme.

The following explanation is a short summary of proline production by propionibacteria. Propionibacteria produce high amounts of proline from Trypticase, a casein hydrolysate. Production of proline does not take place before maximum cell numbers are reached, and autolysis has started. Autolysis releases intracellular enzymes of which the proline peptidases would be most important for proline production. Biosynthesis is not involved even if propionibacteria contain the necessary enzymes. Arginine breakdown and protein hydrolysis may produce small amounts of proline.

How would this affect Swiss cheese ripening? During the time in the warm room, growth of propionibacteria takes place, but at the same time, proteolysis of cheese proteins by enzymes released by the high-temperature bacterial starters occurs. This proteolytic activity leads to accumulation of free amino acids and peptides. However, when propionibacteria reach their maximum numbers, autolysis takes place, and proline and, also, some other amino acids are produced by breakdown of accumulated peptides in the cheese, which occurs even at 3 C.

Further studies are required to observe what propionibacteria do to nitrogen compounds in cheese. One possibility would be to use a fast proline-producing strain and a slow proline-producing strain of propionibacteria, and then deter-

mine if different amounts of proline are produced in the cheeses. To measure autolysis of propionibacteria in Swiss cheese, an enzyme specific for propionibacteria could be measured in a Swiss cheese extract. Proline peptidases has been found in Swiss cheese.

Two possibilities for increased proline production by propionibacteria should be mentioned. The first possibility is to produce a mutant of propionibacteria which has lost the control mechanism for proline biosynthesis. The drawback is, of course, that one does not know how this will affect the other metabolic activities of propionibacteria. The other possibility would be to produce a super-autolytic mutant of propionibacteria, which will autolyze much faster than the parent strain. Higher amounts of proline may then be produced, because of faster release of proline peptidases. Both possibilities may lead to faster flavor development in Swiss cheese.

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ACKNOWLEDGMENTS

I would like to express my sincere thanks to Dr. George W. Reinbold for giving me the opportunity to work for a Ph.D. degree at Iowa State University, for his assistance in choosing an interesting research project and for his guidance and discussion for clarification of the problems.

Thanks also to Dr. Fred D. Williams for many discussions and suggestions about my research.

Thanks should also be extended to Dr. Earl G. Hammond for suggestions about chemical analysis and to Dr. Peter A. Pattee for discussions and advice. Special thanks to Dr. Paul A. Hartman for joining my committee on a very short notice.

For assistance with the statistical analysis I thank Dr. Donald K. Hotchkiss. I would also like to thank Dr. William W. Marion for the use of the amino acid analyzer.

Appreciation is extended to the Agricultural University of Norway for giving me leave, and to Professor Peter Solberg without whose assistance and positive attitude this would not have been possible.

Finally I would like to thank my wife, Kari, for her help, patience and encouragement.

APPENDIX

Table A1. Amino acid composition of Swiss cheese of different quality and Italian-cheeses (μMole/100 g)

Amino acid	Sweet, balanced flavor Swiss cheeses		Sweet, rancid flavor Swiss cheeses		Sweet, unbalanced flavor Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Range
Aspartic acid	66.22	19.42-252.13	74.54	26.31-140.27	80.05	26.36-267.23
Threonine	976.00	521.41-2064.52	897.49	572.52-1201.65	663.31	357.67-919.98
Serine	916.44	403.90-2084.21	923.76	571.36-1252.74	713.32	376.31-930.32
Asparagine	857.55	73.42-2479.94	1111.41	577.33-1674.98	966.26	476.77-1290.13
Glutamic acid	5736.38	2575.20-11148.60	5212.38	3436.00-6687.20	4203.63	2682.40-5119.70
Glutamine	705.31	379.46-1411.72	596.66	295.95-935.34	510.51	362.05-878.29
Proline	4207.94	2145.63-7918.88	3971.05	2361.32-5409.86	2443.33	1567.95-3902.49
Glycine	1207.71	602.03-2242.48	1124.60	703.25-1471.42	973.41	617.59-1299.64
Alanine	2104.23	1103.95-3766.42	2137.11	862.44-3232.49	1517.47	1051.92-2253.26
Valine	2382.52	1256.03-3979.59	2341.51	1313.29-3469.60	1735.30	1193.09-2461.04
Methionine	522.47	242.38-965.90	570.56	348.21-731.80	384.68	290.97-590.60
Isoleucine	1233.25	555.70-2521.15	1284.19	961.34-1404.25	718.57	396.71-1401.89
Leucine	4405.80	2754.18-6508.73	4407.05	2800.83-5993.87	3530.54	2586.00-4725.06
Tyrosine	263.29	54.08-690.45	297.98	30.04-554.00	357.64	222.93-593.87
Phenylalanine	1479.61	984.55-2329.10	1523.86	1002.04-2210.33	1090.64	828.15-1551.39
γ-Amino Butyric acid	52.93	0.00-324.08	51.40	0.00-93.06	3.97	0.00-19.842
Ammonia + Ornithine	2105.62	681.89-3262.92	1951.89	1390.63-2514.59	1471.62	1050.87-2191.63
Lysine	3085.86	1410.34-7029.05	3443.53	2347.43-4269.13	2153.09	1336.20-4034.57
Histidine	822.37	158.55-1610.08	924.41	594.70-1377.15	521.88	308.47-862.43
Arginine	21.42	0.00-94.60	41.77	0.00-114.02	11.18	0.00-19.55
Sum amino acids	33152.87	19512.80-56680.10	32887.11	21315.60-42528.30	24051.37	16006.00-33642.80

Table A1 (Continued)

Amino acid	Low sweetness, unbalanced flavor Swiss cheeses		Not sweet, low flavor Swiss cheeses		Iowa-style Swiss cheeses, unbalanced	
	Average Value	Range	Average Value	Range	Average Value	Range
Aspartic acid	39.32	26.79-50.42	26.52	16.46-38.73	59.85	41.07-98.00
Threonine	706.31	409.76 1063.90	400.96	234.20-561.70	477.40	276.54-621.40
Serine	680.35	418.23-929.06	453.33	153.30-736.21	406.14	224.41-636.19
Asparagine	913.24	497.65-1306.31	733.08	0.00-1352.32	732.30	325.21-1131.21
Glutamic acid	4392.05	3253.00-5360.80	2238.46	1057.10-3137.00	2812.57	1928.30-3367.80
Glutamine	522.58	383.77-665.16	324.79	75.54-591.37	506.61	326.85-960.02
Proline	2242.15	1894.93-3132.34	1380.59	379.65-2382.63	1689.49	1349.63-1993.36
Glycine	958.50	734.21-1162.35	498.40	150.19-748.73	556.88	334.49-711.05
Alanine	1317.24	1054.81-1579.67	888.96	387.55-1456.23	1003.11	641.78-1454.73
Valine	1685.76	1566.13-1901.57	899.26	579.95-1084.31	1165.19	710.65-1538.11
Methionine	403.86	314.36-528.26	232.05	146.23-306.13	375.53	286.95-484.36
Isoleucine	537.16	404.73-671.05	413.99	208.43-640.04	385.70	252.45-650.34
Leucine	3540.12	3093.11-4048.15	2259.49	1172.84-3050.71	2692.68	2043.50-3769.27
Tyrosine	358.49	242.99-549.18	247.26	12.34-403.85	275.11	182.15-481.95
Phenylalanine	1023.23	791.72-1216.23	743.63	419.50-1041.66	857.67	638.22-1158.26
γ-Amino Butyric acid	0.00	n.d.	4.98	n.d. -14.93		n.d. -72.23
Ammonia + Ornithine	1281.05	1121.29-1581.75	941.74	635.60-1299.38	962.79	581.12-1493.61
Lysine	1744.65	1322.45-2229.65	1303.33	541.76-2096.77	1643.94	1155.04-2167.30
Histidine	396.03	314.01-507.58	397.02	88.37-507.58	311.61	275.83-332.50
Arginine	34.09	24.77-41.31	36.48	15.81-76.99	264.33	78.08-504.52
Sum amino acids	22776.14	18929.40-25554.70	14424.32	6275.60-20644.10	17198.84	12204.20-22578.90

Table A1 (Continued)

Amino acid	Iowa-style Swiss cheeses, fresh		Italian cheeses	
	Average Value	Range	Average Value	Range
Aspartic acid	28.20	23.56-35.81	727.74	526.71-1099.14
Threonine	149.18	132.42-178.60	1481.60	1138.50-1757.11
Serine	110.05	88.16-135.42	2653.36	2075.46-3107.29
Asparagine	169.21	150.75-188.68	2125.60	1789.38-2436.71
Glutamic acid	1045.64	772.20-1285.50	9282.60	7746.60-11777.40
Glutamine	130.79	126.34-137.61	555.53	413.71-673.72
Proline	726.44	571.88-868.49	5053.62	4426.00-6085.78
Glycine	196.89	136.43-194.58	1708.38	1548.82-2054.55
Alanine	496.07	411.56-583.67	1871.34	1601.39-2555.19
Valine	421.16	250.92-556.12	3185.82	2596.05-3887.57
Methionine	148.32	102.62-179.61	822.87	667.50-1102.56
Isoleucine	146.49	99.27-158.49	2453.43	2183.02-2917.25
Leucine	1319.56	446.94-1845.64	5417.23	4477.22-6411.39
Tyrosine	134.57	73.33-175.32	q686.69	593.54-771.00
Phenylalanine	364.64	110.78-513.04	1928.25	1521.06-2493.80
γ-Amino Butyric acid	0.00	n.d.	105.19	n.d. -374.99
Ammonia + Ornithine	290.99	186.23-347.44	1323.45	775.95-1811.66
Lysine	459.85	282.92-578.77	3712.45	2147.99-4773.02
Histidine	143.62	79.86-178.21	966.25	570.98-1262.47
Arginine	37.06	36.33-37.50	0.00	n.d.
Sum amino acids	6518.69	4295.40-8158.20	46061.38	39644.60-54661.40

Table A2. Amino acid composition of Swiss cheese of different quality and Italian cheeses in μMole %

Amino acid	Sweet, balanced flavor Swiss cheeses		Sweet, rancid flavor Swiss cheeses		Sweet, unbalanced flavor Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Range
Aspartic acid	0.180	0.069-0.639	0.218	0.109-0.414	0.348	0.093-1.165
Threonine	2.831	2.254-4.058	2.738	2.374-3.548	2.730	2.235-3.186
Serine	2.641	1.810-3.715	2.833	2.424-3.181	2.946	2.351-4.042
Asparagine	2.378	0.296-4.525	3.317	2.708-4.018	4.251	1.417-5.277
Glutamic acid	17.056	12.871-21.683	15.912	15.057-16.186	17.604	15.218-20.071
Glutamine	2.173	1.218-2.931	1.765	1.388-2.199	2.201	1.132-3.547
Proline	12.640	10.996-14.853	11.900	11.164-12.729	10.043	7.599-11.600
Glycine	3.624	2.974-4.441	3.405	3.285-3.632	4.062	3.566-5.249
Alanine	6.381	4.068-9.053	6.320	4.046-7.601	6.295	5.369-6.910
Valine	7.161	6.010-7.809	6.943	6.088-8.158	7.212	6.622-7.701
Methionine	1.600	0.886-2.248	1.737	1.634-1.842	1.598	1.406-1.818
Isoleucine	3.626	2.269-5.083	3.975	3.508-4.510	2.861	2.067-4.167
Leucine	13.550	9.174-19.269	13.463	11.561-16.206	14.826	13.755-16.156
Tyrosine	0.839	0.160-2.373	0.937	0.141-1.815	1.484	0.972-1.862
Phenylalanine	4.634	2.849-7.077	4.618	4.143-5.197	4.565	4.801-5.174
γ-Amino Butyric acid	0.183	0.000-1.184	0.178	0.000-0.430	0.017	0.000-0.087
Ammonia + Ornithine	6.519	2.501-9.025	6.067	5.278-7.691	6.121	4.654-7.547
Lysine	9.295	6.029-14.172	10.654	9.382-11.633	8.659	7.241-11.992
Histidine	2.501	0.558-3.463	2.881	1.756-3.725	2.129	1.513-2.757
Arginine	0.156	0.000-0.260	0.136	0.000-0.268	0.046	0.000-0.079

Table A2 (Continued)

Amino acid	Low sweetness, unbalanced Swiss cheeses		Not sweet, fresh Swiss cheeses		Good Iowa style Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Range
Aspartic acid	0.170	0.142--0.197	0.200	0.149-0.262	0.352	0.221-0.516
Threonine	3.035	2.165--4.189	2.981	2.489-3.732	2.740	2.266-2.997
Serine	2.941	2.209--3.658	2.962	2.443-3.566	2.299	1.839-2.818
Asparagine	4.090	1.960--6.154	3.910	0.000-6.551	4.031	2.665-5.132
Glutamic acid	19.203	17.185--20.978	15.819	15.195-16.844	16.499	14.350-18.077
Glutamine	2.312	2.091--3.042	1.983	1.204-2.865	2.821	2.159-4.252
Proline	9.830	7.896--12.335	8.676	6.050-11.541	10.158	7.272-11.810
Glycine	4.217	3.507--4.932	3.222	2.393-3.646	3.197	2.741-3.743
Alanine	5.769	5.561--6.182	6.356	3.987-8.905	5.694	4.722-6.443
Valine	7.546	6.730--8.274	6.959	5.006-9.241	6.722	5.823-7.430
Methionine	1.758	1.583--2.080	1.768	1.483-2.330	2.193	2.036-2.351
Isoleucine	2.335	2.119--2.643	2.942	2.406-3.321	2.183	1.816-2.880
Leucine	15.563	14.572--16.485	16.573	12.376-18.689	15.735	13.265-16.744
Tyrosine	1.556	1.145--2.163	1.381	0.197-1.956	1.557	1.161-2.134
Phenylalanine	4.477	3.730--4.789	5.479	4.707-6.685	4.982	4.615-5.230
γ-Amino Butyric acid	n.d.	0.000--0.000			0.111	0.000-0.380
Ammonia + Ornithine	5.814	4.415--8.356	7.289	5.444-10.128	5.556	3.871-6.678
Lysine	7.586	6.230--8.780	8.855	7.775-10.157	9.459	7.865-10.909
Histidine	1.736	1.479--1.999	2.350	1.408-4.191	1.887	1.413-2.260
Arginine	0.156	0.097--0.121	0.271	0.077-0.471	1.836	0.346-3.360

Table A2 (Continued)

Amino acid	Fresh, Iowa style Swiss cheeses		Italian cheeses	
	Average	Range	Average	Range
Aspartic acid	0.452	0.332-0.439	1.559	1.161-2.011
Threonine	2.398	1.922-3.083	3.222	2.510-3.600
Serine	1.794	1.241-2.481	5.781	4.575-6.588
Asparagine	2.784	2.123-3.916	4.635	3.944-5.212
Glutamic acid	16.310	15.195-17.977	20.129	17.076-21.607
Glutamine	2.159	1.549-2.990	1.221	0.933-1.699
Proline	11.455	10.404-13.314	10.975	9.984-11.543
Glycine	3.033	2.740-3.183	3.711	3.502-3.907
Alanine	8.475	5.795-13.588	4.040	3.612-4.675
Valine	6.362	5.842-6.817	6.907	5.856-7.423
Methionine	2.294	2.202-2.389	1.777	1.461-2.017
Isoleucine	2.256	2.227-2.311	5.330	5.021-5.584
Leucine	18.829	10.405-23.458	11.759	10.839-14.132
Tyrosine	2.013	1.707-2.183	1.499	1.282-1.699
Phenylalanine	5.162	2.579-6.619	4.183	3.285-4.562
γ-Amino Butyric acid	n.d.	n.d.-n.d.	0.230	0.000-0.810
Ammonia + Ornithine	4.457	4.429-4.777	2.835	1.957-3.352
Lysine	6.991	6.587-7.291	8.101	5.418-10.521
Histidine	2.162	1.859-2.509	2.106	1.440-2.848
Arginine	0.615	0.445-0.873	n.d.	n.d.-n.d.

Table A3. The sum of different amino acid groups of Swiss cheeses of different quality and Italian cheeses (μMole/100 g)

	Sweet balanced flavor Swiss cheeses		Sweet, rancid flavor Swiss cheeses		Sweet, unbalanced flavor Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Range
Acids	5802.61	2604.0-11276.6	5286.92	3471.8-6771.7	4283.68	2708.8-5150.9
Basic	3929.64	1889.8-8639.1	4409.71	3059.2-5760.3	2686.15	16519.9-4913.0
Bitter	13694.09	8806.0-22951.1	14264.28	9820.3-18484.3	10118.83	6953.9-15646.3
Sweet	9412.31	4955.8-18076.5	9054.00	5070.9-11992.1	6311.84	3971.4-9205.6
Bases	6035.26	3438.6-11902.0	6361.60	4698.7-8274.9	4157.76	2702.8-7104.6

	Low sweetness, unbalanced flavor Swiss cheeses		Not sweet, low flavor Swiss cheeses		Iowa-style, balanced flavor Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Range
Acids	4431.37	3279.8--5411.2	2264.98	1073.6-3175.7	2872.42	1969.4-3465.8
Basic	2174.77	1677.7--2768.1	1736.83	646.8-2977.9	2219.87	1819.2-2564.4
Bitter	9319.51	7821.4--10812.2	6300.47	3039.8-8651.9	7596.20	5646.2-10162.4
Sweet	5904.54	4540.5--7428.0	3622.24	1304.9-5252.4	4133.02	2826.8-5057.4
Bases	3455.83	2945.6--3889.3	2678.58	1282.4-4277.2	3182.66	2585.5-4058.1

Table A3 (Continued)

	Iowa-style, fresh Swiss cheeses		Italian cheeses	
	Average Value	Range	Average Value	Range
Acids	1073.83	797.4-1321.3	10010.33	8273.3-12876.5
Basic	640.52	400.3-787.9	4678.70	2719.0-5920.4
Bitter	3026.93	1381.5-4059.7	18350.11	14570.5-20963.8
Sweet	1678.62	1531.0-1569.8	12768.29	11601.9-15559.9
Bases	931.51	586.5-1135.3	6002.15	3494.9-7398.3

Table A4. The sum of different amino acid groups of Swiss cheeses of different quality and Italian cheeses (μMole %)

	Sweet, balanced flavor Swiss cheeses		Sweet, rancid flavor Swiss cheeses		Sweet, unbalanced flavor Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Range
Acids	17.25	13.02-21.87	16.13	15.71-16.44	17.95	15.31-20.22
Basic	11.87	7.83-17.43	13.67	11.91-15.59	10.83	8.83-14.60
Bitter	41.79	32.42-49.47	43.61	41.76-46.07	41.78	39.34-46.51
Sweet	28.12	24.73-31.89	27.20	23.79-28.81	26.07	24.44-28.97
Bases	18.38	13.15-24.10	19.74	17.61-22.04	16.96	14.16-21.12

	Low sweetness, unbalanced flavor Swiss cheeses		Not sweet, low flavor Swiss cheeses		Iowa-style, balanced flavor Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Range
Acids	19.37	17.33-21.18	16.02	15.38-17.11	16.84	14.57-18.41
Basic	9.48	7.90-10.90	11.47	9.70-14.43	13.18	11.36-14.91
Bitter	40.86	36.85-42.68	44.81	41.91-48.44	44.36	41.13-46.27
Sweet	25.79	23.99-29.25	24.20	20.79-26.35	24.09	22.40-25.66
Bases	15.29	13.88-17.84	18.77	15.14-15.32	18.74	17.22-21.59

Table A4 (Continued)

	Iowa-style, fresh Swiss cheeses		Italian cheeses	
	Average Value	Range	Average Value	Range
Acids	16.76	15.53--18.56	21.69	18.24-23.56
Basic	19.77	9.32--10.33	10.21	6.86-13.34
Bitter	44.39	32.16--51.24	39.89	36.75-46.21
Sweet	27.16	2.10-35.64	27.73	25.92-29.66
Bases	14.22	13.65--15.10	13.04	8.82-16.69

Table A5. Differences in ratios between groups of amino acids and between single amino acids in Swiss cheeses of different quality and in Italian cheeses

	Sweet, balanced flavor Swiss cheeses		Sweet, rancid flavor Swiss cheeses		Sweet, unbalanced flavor Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Range
Acids/Basic	1.54	0.89-2.36	1.19	1.05-1.34	1.73	1.05-2.29
Bitter/Sweet	1.51	1.02-2.00	1.61	1.45-1.94	1.61	1.36-1.75
Acids/Bases	0.98	0.59-1.36	0.82	0.74-0.90	1.09	0.73-1.43
Sweet/Acids	1.64	1.41-1.96	1.69	1.46-1.77	1.47	1.23-1.79
Glu/Pro	1.35	1.09-1.57	1.34	1.22-1.46	1.82	1.31-2.64
Thr/Ser	1.10	0.83-1.53	0.97	0.78-1.21	0.94	0.79-1.00
Leu/Iso	3.93	2.19-6.77	3.40	2.82-4.02	5.60	3.37-7.14
Pro/Gly	3.52	2.83-4.43	2.50	3.35-3.80	2.57	1.45-3.25
Phe/Tyr	10.55	2.33-28.16	11.21	2.54-33.35	3.19	2.61-4.20

	Low sweetness, unbalanced flavor Swiss cheeses		Not sweet, low flavor Swiss cheeses		Iowa-style, balanced flavor Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Range
Acids/Basic	2.08	1.67-2.63	1.44	1.07-1.66	1.28	1.08-1.39
Bitter/Sweet	1.59	1.44-1.70	1.88	1.65-2.33	1.85	1.60-2.01
Acids/Bases	1.29	0.97-1.50	0.87	0.74-1.03	0.91	0.81-1.07
Sweet/Acids	1.34	1.15-1.61	1.52	1.22-1.69	1.44	1.37-1.54
Glu/Pro	2.03	1.46-2.66	1.98	1.31-2.78	1.66	1.43-1.97
Thr/Ser	1.02	0.95-1.15	1.05	0.76-1.33	1.21	0.98-1.45
Leu/Iso	6.72	5.81-7.71	5.79	3.99-7.76	7.36	5.80-8.26
Pro/Gly	2.42	1.74-3.52	2.67	2.31-3.18	3.22	2.34-4.04
Phe/Tyr	2.99	2.22-3.41	12.98	2.36-33.99	3.45	2.40-4.27

Table A5 (Continued)

	Iowa-style, fresh Swiss cheeses		Italian cheeses	
	Average Value	Range	Average Value	Range
Acids/Basic	1.72	1.50-1.99	2.34	1.40-3.36
Bitter/Sweet	1.77	0.90-2.32	1.45	1.24-1.78
Acids/Bases	1.18	1.03-1.36	1.79	1.14-2.61
Sweet/Acids	1.60	1.42-1.92	1.28	1.21-1.42
Glu/Pro	1.43	1.35-1.48	1.84	1.48-1.99
Thr/Ser	1.37	1.24-1.55	0.56	0.53-0.59
Leu/Iso	8.39	4.50-10.51	2.20	2.05-2.53
Pro/Gly	3.78	3.35-4.19	2.96	2.79-3.30
Phe/Tyr	2.49	1.51-3.03	2.80	2.54-3.38

Table A6. Differences in volatile fatty acids (μMole/100 g) in Swiss cheeses of different quality, and in ratios of Propionic acid/Acetic acid and Propionic acid/Proline

	Sweet, balanced flavor Swiss cheeses		Sweet, rancid flavor Swiss cheeses		Sweet, unbalanced flavor Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Range
Acetic acid	3997.0	2152.0-5731.0	3873.7	3056.0-4745.0	2908.9	1984.5-3828.0
Propionic acid	4036.4	1944.0-5936.0	4706.4	3566.0-5758.5	2711.4	1901.0-3858.0
Butyric acid	466.5	268.0-665.0	649.5	275.0-788.0	603.6	295.5-841.5
Propionic/Acetic	1.02	0.65-1.39	1.22	1.08-1.45	0.93	0.87-1.01
Propionic/Proline	1.08	0.47-2.71	1.35	0.68-2.27	1.16	0.99-1.29

	Low sweetness, unbalanced flavor Swiss cheeses		Not sweet, low flavor Swiss cheeses		Iowa-style, balanced flavor Swiss cheeses	
	Average Value	Range	Average Value	Range	Average Value	Rance
Acetic acid	2690.6	1898.0-3128.0	2683.8	1525.5-4170.5	2529.1	1286.5-4145.5
Propionic acid	2596.3	1905.0-2939.0	3317.3	1706.0-6259.0	3979.0	2520.5-5696.5
Butyric acid	634.3	276.0-816.0	526.0	368.0-810.5	313.5	280.5-351.0
Propionic/Acetic	0.98	0.84-1.10	1.17	0.72-1.50	1.64	1.30-1.96
Propionic/Proline	1.19	0.94-1.45	6.22	0.72-16.49	2.28	1.87-2.86

Table A6 (Continued)

	Iowa-style, fresh Swiss cheeses	
	Average Value	Range
Acetic acid	1624.8	1308.5-1832.5
Propionic acid	2192.2	1531.0-2595.5
Butyric acid	296.0	275.5-328.0
Propionic/Acetic	1.39	0.88-1.87
Propionic/Proline	3.00	2.68-3.32