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Proteolysis by Streptococcus lactis

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PROTEOLYSIS BY STREPTOCOCCUS LACTIS

by

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TABLE OF CONTENTS

PROTEOLYSIS BY STREPTOCOCCUS LACTIS.

INTRODUCTION

The most conspicuous change which Streptococcus lactis brings about in milk is the formation of the curd resulting from the lactic acid development. This curd does not suggest digestion of the casein as it is ordinarily observed in cultures of the true proteolytic organisms. The appearance of a culture, however, is not a satisfactory basis on which to judge whether or not a particular organism brings about proteolysis in milk and chemical examinations are necessary before an organism can be regarded as non-proteolytic.

The proteolysis brought about by micro-organisms in dairy products is of considerable significance from the standpoint of the production of both desirable and undesirable changes. The degradation of protein is the most important process involved in changing the tough rubbery curd of green cheese to a well ripened product. The proteolytic changes are also of considerable interest because of the probability of the decomposition of the nitrogenous substances in milk, cream, and butter into products having either desirable or undesirable flavors and aromas.

Because of the large numbers of S.lactis organisms present in certain dairy products and the common use of

butter cultures containing these organisms in the manufacture of butter and cheese, a study of their action on the proteins of milk is of considerable importance.

STATEMENT OF THE PROBLEM

The work herein reported was undertaken to determine whether or not the commonly isolated strains of S.lactis are able to attack the protein of milk in a significant manner, and to show the relationship of such proteolytic strains to the desirable or undesirable flavors and aromas of butter.

REVIEW OF LITERATURE

Orla-Jensen (1904) isolated two strains of Bacterium lactis acidi from Emmental cheese and found that when they were grown for 3 months in milk with added chalk there was considerable proteolysis as shown by analyses of the cultures for soluble, amino and ammonia nitrogen. One of the strains fermented more sugar and decomposed more protein at 35°C. than at room temperature. As a rule the decomposition of protein was increased when the time of holding was increased.

von Freudenreich and Thoni (1905) made small experimental cheese from milk procured under aseptic conditions and to which different lactic acid bacteria had

been added. The cheese made by the addition of Bact. lactis acidi had a characteristic Emmental cheese flavor and odor and there was an increase in the soluble nitrogen content. They found as high as 30.35 per cent of the total nitrogen in a soluble form in old cheese (6 1/2 months) that was made from a large amount of milk produced under very careful conditions and to which a culture of Bact. lactis acidi had been added.

Barthel (1915) compared the Van Slyke method for determining amino nitrogen with that of precipitation with phosphotungstic acid and showed that the former method is better in securing accurate results. He isolated S.lactis strains from starter, milk, and various cheeses and found they decomposed more protein at 14 to 20°C. than at 36°C. when grown for from 2 to 4 months in milk to which $CaCO_3$ had been added in amounts sufficient to neutralize the acid that could be formed. Most of the strains of S.lactis fermented more lactose at lower temperature than at 36°C. One strain isolated from commercial milk, when grown at room temperature in chalk milk for 5 months, decomposed casein in an amount quite comparable with that decomposed by lactobacilli grown at 36°C. under the same conditions.

In comparing the proteolysis by Streptococcus erysipelatis with that by Streptococcus lacticus in beef infusion bouillon, Itano (1916) used Sorensen's formol titration method as the criterion of protein cleavage.

It seems from his results that S.lacticus possesses the power of breaking down protein.

From the work of Ott de Vries (as reported by Barthel and Sandberg (1919)) it appears that S.lactis strains isolated from milk, starter or cheese have a varying ability of decomposing the casein in milk. He followed the same procedure as that employed by Barthel except that Ott de Vries substituted the phosphotungstic acid method for Van Slyke's method of determining amino nitrogen. Ott de Vries observed that the soluble nitrogen fluctuated between 0 and 20.1 per cent and the decomposed nitrogen (amino nitrogen) between 0 and 5.5 per cent of the total nitrogen when the tested cultures were incubated at room temperature in chalk milk for from 2 to 3 months.

Barthel and Sandberg (1919) in continuing the study of the casein decomposing ability of S.lactis begun by Barthel, classified the decomposition products into soluble, trialbumin, peptone and amino nitrogen in order to show the different stages of protein cleavage. The amounts of soluble nitrogen formed in milk by 22 newly isolated strains, partly from milk and partly from starters, varied from 0 to 23.21 per cent of the total nitrogen. Nine strains isolated from a sample of milk were on the whole strong proteolyzers while 2 out of 4 strains isolated

from cheese had poor casein decomposing properties. Only trialbumin or peptone nitrogen and not amino nitrogen was found in milk cultures of the strains having poor proteolyzing powers. Both poor and strong casein splitting strains were found in the same sample of starter. The protein cleavage ability was constant for the same strain under varied unfavorable conditions and the accumulation of the soluble nitrogen produced had no effect on the inherent casein decomposing ability of the strain. The significance of lactococci in ripening experimental Emmental cheese made by the use of alum in place of rennet and with the addition of chalk was shown by the flavors and aromas and also by the various nitrogen decomposing products. The hydrogen ion concentration in the cheese was claimed to be almost similar to that ordinarily found in hard cheese.

Orla-Jensen (1919) isolated large numbers of strains of S.lactis and Streptococcus cremoris from milk, butter, cheese, buttermilk, whey, starter and different fermented milks, all secured from various sources, and studied their proteolytic action on milk and Witte's peptone broth. By inoculating these strains into milk with added chalk and incubating at 30°C. for a period of one month, he found that from 0 to 20.4 per cent of the total nitrogen was changed into soluble forms and that the decomposed nitrogen determined by the phosphotungstic

acid method varied from -0.9 to 10.1 per cent of the total nitrogen. In Witte's peptone the decomposed nitrogen varied from 6.1 to 16.5 per cent and the formol titratable nitrogen fluctuated between 4.0 to 15.2 per cent. No relationship was found between the proteolytic powers of the strains and the rate of curdling the milk at 30°C.; many poor proteolyzing strains curdled milk very rapidly.

Virtanen (1923) incubated for one week at room temperature milk cultures of S.lactis strains isolated partly from milk and partly from Emmental cheese and observed that the maximum acidity produced by the different strains varied considerably. By inoculating chalk milk with the strains and determining the casein decomposition by the method used by Barthel he found that there was no correlation between the maximum acidity produced and the degree of casein cleavage. Many strong acid forming S.lactis strains did not decompose the casein in appreciable amounts.

A S.lactis culture studied by Spitzer, Parfitt and Epple (1927) caused a slight increase in various groups of protein decomposition products when grown in milk or in an unsalted synthetic butter made by adding skimmilk to butter fat.

Peterson, Pruess and Fred (1928) in their study of the proteolytic action of certain lactic acid bacteria, included a strain of S.lactis and found a decomposition of

protein in three different media used. Formation of non-protein, amino and ammonia nitrogen was used as a measure of proteolysis. The cultures of the S.lactis strain were incubated for various lengths of time and in a majority of cases an increase in amino nitrogen was noted.

S.lactis strains secured from different laboratories and culture collections by Hucker (1928) showed marked variations in their ability to decompose the nitrogenous constituents of milk to amino acids (determined by the Van Slyke method). In the trials the milk cultures (without $CaCO_3$) were incubated at the optimum temperature (25°C.) for 17 to 19 days. Four out of 12 strains investigated, although growing readily in milk, showed little or no increase in amino nitrogen, while the remaining 8 cultures attacked the protein giving a real increase in amino nitrogen. Out of 4 starters included in the study one behaved just like the control sample while the other three showed some proteolysis.

By passing a stream of hydrogen or carbon dioxide through flasks of chalk milk inoculated with starters of commercial origin or from different dairies, Barthel and Sadler (1928) were able to control the organisms other than lactococci and aroma bacteria. The milk cultures of the starters thus treated were held at

room temperature for 2 months and analyzed for soluble and amino nitrogen. It was found that the starters possessed a much greater ability to form amino nitrogen from casein than pure cultures of lactococci and aroma bacteria while there was practically no difference between the soluble nitrogen formed by starters and by the pure cultures.

Anderegg and Hammer (1929) isolated a number of S.lactis cultures from untreated, filtered and clarified milk, clarifier slime, sweet and sour cream, butter cultures and cheese and tried out their proteolytic activity in milk with and without added $CaCO_3$, using, as a rule, 14 days incubation at room temperature. In general there was definite proteolysis by cultures coagulating milk rapidly while the slow curdling cultures apparently did not decompose the protein. The associated organisms, Streptococcus citrovorus and Streptococcus paracitrovorus, did not show any proteolytic activity in milk while the butter cultures studied definitely showed proteolysis. On the whole protein cleavage was more pronounced when $CaCO_3$ was added to the milk as a neutralizing agent than when it was not. Sterile lactic acid added to milk and held at room temperature did not increase its soluble nitrogen content which shows that the proteolytic change is not due to the lactic acid developed by S.lactis. The addition of peptone seemed to

retard the proteolytic activity rather than to accelerate it. It was evident that the degradation of protein was carried to the amino acid stage, because there was a corresponding increase in amino nitrogen, as determined by the Van Slyke method, when there was an increase in soluble nitrogen.

METHODS USED

Proteolysis in milk was measured by determining the soluble and amino nitrogen in milk in which the organisms had grown. Sometimes $CaCO_3$ was added to the milk to neutralize the acid produced and thus permit more extended activity of the organisms. The decomposition of protein in butter was measured by running soluble and amino nitrogen determinations on the filtrate secured from the non-fatty material of the butter.

Methods for Trials in Milk

Some of the organisms used in the experiments herein reported were selected from the stock cultures maintained by the Dairy Industry Department of Iowa State College. About 120 strains of S.lactis were isolated from milk and cream, both sweet and sour, and from butter cultures by plating on whey agar and picking the colonies. Each sample of milk and cream came from an individual

producer. The butter cultures employed were those which were being carried by the Dairy Section of the Iowa Agricultural Experiment Station.

All S.lactis cultures used in the trials were kept active by frequent transfers through a series of tubes of sterile litmus milk. Vigorous cultures (either 24 or 48 hours old) were employed for inoculating the milk in the proteolysis trials.

Pint milk bottles were used as containers. By giving close attention to the rate of heating the breakage due to the thick wall was negligible.

For the trials in sterile milk 200 cc. of skim-milk were introduced into each bottle which was then cotton plugged, weighed, and the weight recorded on a gummed label that was fastened to the cotton plug. In the trials where an attempt was made to neutralize the lactic acid produced by the organisms, about 8 gms. of finely powdered $CaCO_3$ and a few pieces of glass (to make mixing easy) were put into each bottle before introducing the skimmilk. As a rule the bottles of milk for each group of trialswere sterilized in one lot. They were first placed in flowing steam in the autoclave with the door open for about five minutes so that they would be heated slowly. After closing the door the pressure was increas-ed gradually to 15 pounds and then maintained for 22 minutes.

For the trials in pasteurized milk the bottles were prepared as for sterile milk and were pasteurized by placing them in a water bath with a false bottom. The water was brought to a boil slowly on a gas stove and allowed to boil for about 2 or 3 minutes after which the heat was shut off. The milk was left for 30 minutes and then cooled by running cold water into the warm water surrounding the bottles.

In most trials a loop of a vigorous culture was tranferred to each bottle containing 200 cc. of skim-milk. In some instances where the rate of coagulating milk by different strains was to be observed closely, 1 cc. portions of the cultures were placed in 9 cc. lots of sterile water and, after vigorous shaking, measured portions of this diluted material were employed for inoculating the milk.

In general all cultures were incubated at room temperature except where the proteolytic activity of an organism was compared at different temperatures. As a rule the curdling of the milk was observed at 24 hour intervals after inoculation. The cultures containing added $CaCO_3$ were vigorously shaken daily.

The soluble portion was recovered for analysis by flocculating the insoluble contents of the bottle by means of acetic acid and heat. At the end of the incuba-

tion period the loss of water due to evaporation was made up by adding water to each bottle until the weight reached 1 gm. less than the original. In the absence of $CaCO_3$ 1 cc. concentrated acetic acid was added to each bottle and the bottle immediately given a thorough shaking. The bottles were then placed in a water bath and heated slowly to 60°C. after which they were removed and cooled. In the presence of $CaCO_3$ the procedure was the same except that 1 cc. acetic acid was added to the milk after decanting as completely as possible from each bottle, care being taken to exclude the glass and the carbonate.

In filtering, the entire contents of a bottle were thrown on a filter paper and the first turbid filtrate was returned for a second filtration. The filtrates were then passed through Berkefeld filters in order to make them uniformly clear. This procedure was simplified by attaching a row of suction flasks to a single water pump.

For the soluble nitrogen determinations 25 cc. of the filtrate were transferred to a 500 cc. Kjeldahl flask and digested with 25 cc. of concentrated H_2SO_4, about 5 gms. of Na_2SO_4, a small piece of copper wire and about 2 gms. of CCl_3COOH. The CCl_3COOH was included in order to limit the foaming which was considerable in most of the trials. The distillates were collected in fifth normal H_2SO_4 and back titrations made with tenth normal

NaOH using alizarin as an indicator.

The amino nitrogen was determined by the Van Slyke method, using 10 cc. of the filtrate and retarding the foaming with 1 cc. butyl alcohol.

In the majority of the trials the duplicate determinations were made on the same filtrate but in some instances, it was thought advisable to inoculate the cultures in duplicate and then run only one determination on each filtrate. The results in the latter case, therefore, represent the average of two determinations made on two different filtrates.

In every trial control bottles of milk were included in the series and the results are expressed as increases over this control sample. Sometimes the values for both soluble and amino nitrogen are negative; these represent instances in which there was less soluble or amino nitrogen in the filtrate from milk in which an organism had developed than in the filtrate from the control.

Methods For Trials In Butter

In studying the proteolytic activity of S.lactis in butter, several lots of butter were made under various conditions. In general two procedures were followed. In one, small 1 gallon glass churns (in each of which 4 pounds of cream could be churned) were used, and the butter was

worked with wooden paddles in small enamelled pans. All the equipment employed in this procedure was of such a nature that, when desired, it could be sterilized in an autoclave. The butter made was packed in 1 pound paraffined paper cartons and stored at various temperatures. In the second procedure the butter was made under practical conditions in the creamery. For this about 250 pounds of cream were pasteurized at 62.8°C. (145°F.) for 30 minutes in a 600 pound vat and, after cooling to 21.1°C. (70°F.), half of the cream was withdrawn and placed in a second vat of the same capacity. A culture prepared by growing a selected strain of S.lactis in sterilized or pasteurized skim-milk was added to each vat and the cream then cooled down to 4.4°C. (40°F.). After holding from 2 to 3 hours the two lots of cream were churned. For this, Cherry Junior Perfection churns (in each of which 75 pounds of butter could be churned) were used. Part of the butter was packed in 12 oz. paraffined paper cartons and part in 10 pound tubs. Different storage temperatures were used. After holding for various lengths of time the butter was analyzed for total, soluble and amino nitrogen. The tub butter was analyzed also for moisture and salt.

For the nitrogen determinations 180 gms. of butter were melted in a beaker by heating in a water bath,

the temperature of the water never being allowed to go higher than 60°C. Usually from 2 to 3 hours elapsed before the fat was satisfactorily separated from the other material. As much as possible of the clear fat was decanted, care being taken to retain all the non-fatty material. The contents of the beaker were than transferred to a separatory funnel, and by repeated washings with warm water the non-fatty material was separated from the remaining fat and collected in a 300 cc. volumetric flask. Additional water was introduced to bring the contents of the flask up to the 300 cc. mark.

For the total nitrogen determinations 50 cc. (in duplicate) of the diluted material were used. The remaining 200 cc. were divided into two equal portions (as duplicates) and were slightly heated in a water bath. In the case of unsalted butter, 3 cc. of dilute (1 to 10) acetic acid were used to flocculate the insoluble constituents of each 100 cc. portion. After standing for a few minutes the 100 cc. portions were cooled and filtered through paper. From the filtrate which was generally very clear, 25 and 10 cc. portions were taken for determinations of the soluble and amino nitrogen respectively.

In the case of salted butter the attempts to flocculate the insoluble constituents of the diluted non-fatty material by means of acetic acid were

unsuccessful. Various other reagents such as solutions of $EgSO_4$ and $CaCl_3$, Millon's reagent, and a solution of HgI_2, made by mixing 33.2 gms. of KI with 13.5 gms. of $HgCl_3$, 20 cc. of acetic acid and 640 cc. of water, were tried. Some of these were very effective in flocculating the insoluble constituents and making filtration easy, but comparisons of the soluble and amino nitrogen values for the filtrates secured with the values for the filtrates obtained, with difficulty, by means of acetic acid, showed that the nitrogen distribution had been greatly influenced. However, $AlCl_3$ was found to be well adapted to flocculating the insoluble material without affecting the nitrogen distribution. As in the case of unsalted butter, 100 cc. portions from salted butter were used; these were slightly warmed and to each, 3 cc. of 0.1 molar solution of $AlCl_3$ were added in 1 cc. portions, with vigorous shaking after each addition. As a rule 3 cc. of $AlCl_3$ solution were enough to flocculate the curd but in a few instances it was necessary to add 1 or 2 cc. more. After the addition of $AlCl_3$ the 100 cc. portions were allowed to stand for a few minutes and then cooled and filtered through paper.

Sometimes the filtrates from both unsalted and salted butter were slightly turbid. In such cases

the filtrates were clarified by passing through Berkefeld filters.

The procedure employed was the same as that used for milk except that CCl_3COOH was left out as there was no tendency to foam. The amino nitrogen was determined by the Van Slyke method.

The results obtained are expressed as mgms. of soluble or amino nitrogen per 10 cc. filtrate. In order to make them more comparable these values were adjusted to a total nitrogen content of 4.2 mgms. per 10 cc. of the diluted non-fatty material; this value was taken as a standard because it represents the average nitrogen content of the diluted non-fatty constituents of a considerable number of samples of butter.

For moisture determinations 10 gms. of butter were taken from a composite sample made up from several cores obtained with a trier. The butter was weighed on a moisture scale and reweighed after driving off the moisture over a low flame.

For each salt determination the residue from the moisture test was transferred to a separatory funnel and, after repeated washings with warm water, the solution was separated from the fat and collected in a 250 cc. volumetric flask. Additional water was introduced to bring the contents of the flask up to the 250 cc. mark.

The per cent salt was calculated after titrating 25 cc. of the salt solution with $AgNO_3$ using K_2CrO_4 as an indicator.

RESULTS OBTAINED

Results of Trials in Milk

Proteolysis by S.lactis after 7 days.

For the most part the studies reported by various investigators on the proteolysis by S.lactis have been carried out with long incubation periods in milk containing added $CaCO_3$. In order to determine whether or not the protein compounds of milk are decomposed by S.lactis in a shorter time in the absence of $CaCO_3$, a trial was made with six selected strains. The cultures were incubated in sterile milk for 7 days at room temperature and the soluble and amino nitrogen determined.

The data given in Table 1 show the general trend of the results obtained. Definite increases in the soluble and amino nitrogen occurred with the two strains which curdled milk rapidly while the slow coagulators gave either negative values or only very slight increases.

From the results it is evident that proteolysis by S.lactis does not require extended incubation periods but may occur in as short a time as 7 days without the addition of $CaCO_3$ to the milk. The data further suggest

TABLE 1

PROTEOLYSIS BY S.LACTIS AFTER 7 DAYS.

ROOM TEMPERATURE

Strains of S.lactis	General rate of coagulation	Increase (over control) per 10 cc. filtrate	
		Soluble N. mgms.	Amino N. mgms.
18	rapid	1.288	0.545
22	slow	-0.252	0.027
53	slow	-0.252	0.109
67	rapid	2.072	0.872
71	slow	-0.448	0.054
96	slow	-0.028	0.163

a correlation between the proteolytic activity of S.lactis and its rate of coagulation at room temperature.

Proteolysis by S.lactis after different incubation periods.

In order to secure additional information on the proteolysis at different stages in the development of S.lactis cultures, two strains, one a rapid and the other a slow coagulator, were grown in sterile milk at room temperature and soluble and amino nitrogen determinations made after periods varying from 12 hours to 9 days.

The results secured are given in Table 2. Culture 55 which curdled milk rapidly broke down the proteins of milk in as short a time as 1 1/2 day without the addition of $CaCO_3$ to the milk, the amino nitrogen value secured after 12 hours suggests proteolysis but the soluble nitrogen value does not. In general with an increase in the incubation period there was an increase in soluble and amino nitrogen. With culture 58, which coagulated milk slowly, there was no evidence of proteolysis even after 9 days.

The data given in Table 2, show that S.lactis may definitely proteolyze milk without added $CaCO_3$ in as short a time as 1 1/2 day. With the two cultures used the correlation between the proteolysis

TABLE 2

PROTEOLYSIS BY S. LACTIS AFTER D

PERIODS* ROOM TEMPERATU

Strains of S.lactis	General rate of coagulation	Increase (over control						
		12 hours		1 1/2 day		2 days		3
		Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.	Sol N mg
55	rapid	-0.207	0.186	0.448	0.405	1.540	0.531	1.
58	slow			-0.224	-0.027	-0.476	-0.079	-0.

* The milk analysed was curdled in s

E 2

AFTER DIFFERENT INCUBATION

PERATURE.

control) per 10 cc. filtrate after

	3 1/2 days		5 days		7 days		9 days	
ino N. ms.	Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.
531	1.204	0.656	1.904	0.653	1.960	0.858	2.184	0.724
079	-0.448	0.000	-0.616	0.108	-0.700	0.107	-0.168	0.000

ed in all cases.

and the rate of coagulation at room temperature was again evident.

Proteolysis by butter cultures
in sterile milk after 3 and 6 days.

Since S.lactis is one of the main organisms in butter cultures and since butter cultures are widely used in dairy products for the development of flavors and aromas, the effect of such cultures on the proteins of milk was studied. Sterile milk, with and without $CaCO_3$, was used and the cultures were incubated at room temperature for 3 and 6 days.

Table 3 presents the data obtained. The three butter cultures used showed definite proteolysis in milk without $CaCO_3$ even after 3 days. Proteolysis was more pronounced when $CaCO_3$ was present than when it was not. With $CaCO_3$ it was greater after 6 days than after 3 but without $CaCO_3$, the longer incubation did not definitely increase the proteolysis.

From the results it is evident that butter cultures proteolyse milk rapidly and that proteolysis is increased by the presence of $CaCO_3$. Since S.lactis proteolyzes milk it may be the cause of the protein decomposition in butter cultures.

TABLE 3

PROTEOLYSIS BY BUTTER CULTURES IN STERILE MILK AFTER 3 AND 6 DAYS.

ROOM TEMPERATURE

Butter cultures	Increase (over control) per 10 cc. filtrate after							
	3 days incubation				6 days incubation			
	Without $CaCO_3$		With $CaCO_3$		Without $CaCO_3$		With $CaCO_3$	
	Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.
122	1.120	0.540	4.032	1.890	0.504	0.756	4.676	3.645
144	1.540	0.702	3.192	1.674	1.008	0.675	3.612	2.565
146	1.370	0.756	3.338	1.134	0.952	0.648	3.410	2.187

Proteolysis by butter cultures in sterilized and pasteurized milk just curdled.

Butter cultures are generally used in dairy products a short time after their coagulation and, accordingly, soluble and amino nitrogen determinations were made on cultures that had just curdled. Values were secured for six butter cultures in sterilized milk and for four in pasteurized milk; $CaCO_3$ was not used. All cultures were incubated at room temperature. The cultures in pasteurized milk were coagulated in about 12 hours while those in sterilized milk took about 17 hours to curdle.

The results are given in Table 4. The values indicate clearly that there is definite proteolysis in freshly coagulated butter cultures grown in sterilized or pasteurized milk. Three out of the four cultures for which values were obtained in both kinds of milk gave slightly more protein decomposition in sterilized milk than in pasteurized milk, but the differences are negligible.

TABLE 4

PROTEOLYSIS BY BUTTER CULTURES IN STERILIZED AND PASTEURIZED MILK JUST CURDLED. ROOM TEMPERATURE.

Butter cultures	Increase (over control) per 10 cc. filtrate			
	Sterilized milk		Pasteurized milk	
	Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.
122	0.896	0.648	0.728	0.289
144	1.176	0.540	1.036	0.459
146	0.644	0.324	0.812	0.364
182	0.952	0.567	0.700	0.324
183	1.120	0.540	-	-
185	1.036	0.567	-	-

The data show that proteolysis has already occurred in butter cultures at the time they are ordinarily used in dairy plants.

Proteolysis by butter cultures in pasteurized milk held for varying periods.

In order to obtain additional information on the proteolysis at different stages in the development of butter cultures, trials were carried out in pasteurized milk at room temperature using cultures which showed differences in flavor and aroma. Two experiments were carried out using holding periods varying from 14 to 86 hours; in one both soluble and amino nitrogen were determined, while in the other only amino nitrogen values were secured. The soluble and amino nitrogen determinations on the controls were made only at the end of 14 hours (immediately after the coagulation of butter cultures) and the values secured were applied to all the analyses.

The data are given in Table 5. It is evident that the proteins of pasteurized milk are decomposed by butter cultures after as short a time as 14 hours. With butter cultures 122 and 144 the proteolysis was definitely increased with an increase in the incubation period while with butter cultures 10, 23 and 103 it was not.

The results again indicate that butter cultures

TABLE 5

PROTEOLYSIS BY BUTTER CULTURES IN PASTEURIZED MILK HELD FOR VARYING PERIODS. ROOM TEMPERATURE.

EXPERIMENT A

Butter cultures	Increase (over control) per 10 cc. filtrate after 14 hours: Soluble N. mgms.	14 hours: Amino N. mgms.	38 hours: Soluble N. mgms.	38 hours: Amino N. mgms.	86 hours: Soluble N. mgms.	86 hours: Amino N. mgms.
122	0.224	0.162	0.952	0.378	1.120	0.378
144	0.924	0.270	1.120	0.405	1.176	0.486

EXPERIMENT B

Butter cultures	Increase (over control) in amino N. per 10 cc. filtrate after 14 hours mgms.	36 hours mgms.	60 hours mgms.	84 hours mgms.
10	0.325	0.464	0.352	0.354
23	0.244	0.136	0.027	0.000
103	0.244	0.218	0.302	0.245
122	0.298	0.300	0.352	0.354
144	0.135	0.164	0.217	0.300

have already undergone proteolysis at the time they are usually used in dairy plants.

Proteolysis by the organisms associated with S.lactis in butter cultures.

In addition to S.lactis, butter cultures contain other organisms (S.citrovorus or S.paraeitrovorus) which are mainly responsible for the development of flavors and aromas and, accordingly, their proteolytic activity in milk was studied. Six different strains, including both types of associated organisms, were used and the cultures were grown in sterile milk (without added $CaCO_3$) at room temperature for 7 days after which the soluble and amino nitrogen were determined.

Table 6 presents the results secured. Although in most cases both the soluble and amino nitrogen were slightly increased by the organisms, the increases were much less than those secured with butter cultures or proteolytic strains of S.lactis.

Since both S.lactis and butter cultures very definitely proteolyse milk and the associated organisms produce only slight changes in the soluble and amino nitrogen, it seems probable that the protein decomposition in butter cultures is due primarily to S.lactis.

TABLE 6

PROTEOLYSIS BY THE ORGANISMS ASSOCIATED WITH S.LACTIS IN BUTTER CULTURES.

ROOM TEMPERATURE.

Strains of associated organisms	Increase (over control) per 10 cc. filtrate	
	Soluble N. mgms.	Amino N. mgms.
27A	0.280	0.000
27C	0.196	0.081
33A	0.264	0.116
33B	0.112	0.081
34B	0.220	0.109
38A	0.252	0.109

Comparison of proteolysis by butter cultures and by lactic acid.

The prominent change produced in milk by butter cultures is the formation of lactic acid and, accordingly, the possible effect of this acid on the milk proteins becomes of importance. In order to secure information on this point a trial was carried out in which the action of added sterile lactic acid on the proteins of milk was compared with the action of a butter culture, no $CaCO_3$ being used. Lactic acid (U.S.P. VIII) was sterilized and added to the milk in quantities sufficient to bring the final acidities to 1 per cent and 2 per cent. Both pasteurized and sterilized milk were used. The incubation was at room temperature, 12 hours for the pasteurized milk and 3 days for the sterilized milk.

The results are presented in Table 7. The data show that with added lactic acid the increases in soluble and amino nitrogen were negligible and much less than those where butter culture was used. Increasing the amount of acid added did not increase the proteolysis.

Apparently the addition of lactic acid to the extent of 2 per cent did not appreciably proteolyze pasteurized or sterilized milk at room temperature. It, accordingly, appears that the lactic acid produced in

TABLE 7

COMPARISON OF PROTEOLYSIS BY BUTTER CULTURE AND BY LACTIC ACID.

ROOM TEMPERATURE

	Increase (over control) per 10 cc. filtrate			
	Pasteurized milk Incubation period 12 hours		Sterilized milk Incubation period 3 days	
	Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.
Butter culture 122	1.120	0.348	1.036	0.855
1 per cent lactic acid	0.084	0.000	0.056	0.082
2 per cent lactic acid	0.084	0.000	0.000	0.110

milk by the butter cultures is not responsible for the proteolytic change which accompanies their growth in milk.

Relation of air supply to proteolysis by S.lactis and butter cultures.

Since the rate of acid development by S.lactis or butter cultures is sometimes influenced by the air supply and since the rapid coagulating cultures are the ones which generally bring about proteolysis, a trial was carried out to study the relation of air supply to proteolysis by S.lactis and butter cultures in pasteurized milk. The method employed for varying the air supply consisted of using 150 cc. of milk in each of two types of containers, 1 liter cotton-plugged Erlenmeyer flasks and 6 ounce glass-stoppered bottles. The Erlenmeyer flasks had very thin layers of milk while the glass-stoppered bottles were almost full. The milk in the flasks was pasteurized by exposing it to flowing steam for 40 minutes in an autoclave while that in the bottles was heated in a water bath as usual.

Five strains of S.lactis varying in the rate of coagulation and proteolysis, and five butter cultures, showing differences in flavor and aroma, were employed. The cultures were incubated at room temperature and amino nitrogen determinations made immediately after coagulation

(14 to 20 hours).

The data secured are given in Table 8. The cultures which showed definite proteolysis gave somewhat lower values than those obtained in previous trials, probably because the determinations were made immediately after coagulation. However, cultures showing definite proteolysis with an abundant air supply also proteolyzed milk to about the same extent with a restricted air supply. On the other hand cultures which showed questionable proteolysis with an abundant air supply also gave negative values or very slight increases with a restricted air supply.

The results, on the whole, indicate that the air supply apparently does not influence the proteolytic change brought about by S.lactis or butter cultures.

Influence of added peptone or alanine on the proteolytic action of S.lactis cultures.

The influence of the addition of 0.1 per cent peptone or alanine to milk on the proteolysis by S.lactis was studied using 13 strains whose proteolytic activities were known from previous trials. The cultures were incubated in sterile milk without added $CaCO_3$ at room temperature for 7 days and the soluble and amino nitrogen determined.

TABLE 8

RELATION OF AIR SUPPLY TO PROTEOLYSIS BY *S.LACTIS* AND BUTTER CULTURES.

ROOM TEMPERATURE

Cultures used	Increase (over control) in amino N. per 10 cc. filtrate.	
	Under abundant air supply	Under restricted air supply.
	mgms.	mgms.
S.lactis 18	0.265	0.210
S.lactis 55	0.632	0.522
S.lactis 58	0.027	-0.055
S.lactis 80	0.000	-0.055
S.lactis 103	0.495	0.522
Butter culture 23	0.162	0.054
Butter culture 122	0.324	0.297
Butter culture 191	0.270	0.208
Butter culture 193	0.243	0.235
Butter culture 198	0.243	0.216

Table 9 presents the results obtained. In general, with cultures showing definite proteolysis the addition of peptone or alanine decreased the soluble nitrogen values while with those which showed no proteolysis the negative values were increased. On the other hand the retarding effect of peptone or alanine would be questioned from the amino nitrogen values, because sometimes decreases and sometimes slight increases were obtained.

Observations (which are not included in the data) on the rate of coagulation of the cultures grown in milk to which peptone or alanine had been added, indicated that, in general, the cultures coagulated the milk more rapidly with the addition than without.

In general the data show that the addition of peptone or alanine to the extent of 0.1 per cent did not accelerate but, on the other hand, rather retarded the proteolytic activity of S.lactis.

Comparison of the proteolytic action and rate of coagulation of S.lactis.

In order to study further the correlation between the proteolysis and the rapid coagulation of milk a considerable number of S.lactis cultures were investigated. One of the things in mind was to secure a

TABLE 9

INFLUENCE OF ADDED PEPTONE OR ALANINE ON
THE PROTEOLYTIC ACTION OF S.LACTIS CULTURES
ROOM TEMPERATURE

Strains of S.lactis	General rate of coagulation	Increase (over control) per 10 cc. filtrate with					
		No addition		0.1 per cent peptone added		0.1 per cent alanine added	
		Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.	Soluble N. mgms.	Amino N. mgms.
18	rapid	0.448	0.367	-	-	0.056	0.337
55	rapid	1.652	0.587	0.700	0.587	0.638	0.527
58	slow	-0.756	-0.106	-1.036	0.113	-0.868	0.080
67	rapid	-	0.720	1.316	0.614	-	-
71	slow	-0.728	-0.106	-1.036	0.186	-0.868	0.186
55	rapid	1.624	0.783	1.260	0.594	1.288	0.228
101	rapid	1.148	0.378	0.784	0.216	0.840	0.243
102	rapid	1.232	0.351	1.092	0.378	0.784	0.297
24	rapid	0.952	0.513	0.876	0.324	0.924	0.270
74	slow	-0.028	0.189	-	0.027	-	0.054
103	rapid	2.716	0.863	1.904	0.886	2.380	0.806
104	slow	-0.028	0.139	-0.420	0.250	-0.504	0.222
105	slow	0.112	0.055	-0.168	0.306	-0.252	0.055

rapid coagulating non-proteolytic S.lactis strain which could be used in developing a butter culture coagulating milk rapidly with no proteolysis. The cultures studied were isolated from butter cultures, sweet and sour cream and from raw milk; the milk was usually held over night to permit considerable bacterial growth. The trials were carried out in sterile milk without added $CaCO_3$ and the cultures were incubated at room temperature for 7 days. Only the amino nitrogen was determined because the previous trials had shown that whenever there was an increase in amino nitrogen there was a corresponding increase in soluble nitrogen.

The results obtained, together with the general rate of coagulation at room temperature are given in Table 10. Each source is designated by a letter and the different numbers indicate different isolations. The data show that some of the cultures caused a very definite proteolysis, while others did not. A comparison of the proteolytic activity of each organism with its rate of coagulation at room temperature indicates that, as a rule, the cultures which caused a definite proteolysis were rapid coagulators, and that with those causing little or no increase in amino nitrogen the rate of coagulation was slower but showed considerable variation. Among the non-proteolytic cultures, B_1, G_4, K_5, M_3, N_5, O_3, Y_4, BB_3

TABLE 10

COMPARISON OF PROTEOLYTIC ACTION AND RATE OF COAGULATION OF S.LACTIS

ROOM TEMPERATURE

Strains of S.lactis	Increase (over control) per 10 cc. filtrate. amino N. mgms.	General rate of coagulation days	Strains of S.lactis	Increase (over control) per 10 cc. filtrate amino N. mgms.	General rate of coagulation days
A1	0.000	4	G5	0.354	1
A2	-0.054	4	G6	0.463	1
A3	-0.054	4	H1	0.490	1
B1	0.108	2	H2	0.408	1
B2	0.406	1	H3	0.435	1
B3	0.379	1	H4	0.517	1
B4	0.433	1	I1	0.408	1
C1	0.460	1	I2	0.517	1
C2	0.433	1	I3	0.000	3
C3	0.027	4	I4	0.027	3
D1	0.433	1	J1	0.463	1
D2	0.519	1	J2	0.544	1
E1	0.492	1	J3	0.435	1
E2	0.464	1	K1	0.544	1
E3	0.519	1	K2	0.544	1
E4	0.000	3	K3	0.435	1
F1	0.464	1	K4	0.381	1
F2	-0.054	4	K5	0.108	2
G1	0.272	1	K6	0.354	1
G2	0.408	1	L1	0.656	1
G3	0.299	1	L2	0.705	1
G4	-0.027	1	L3	0.550	1

TABLE 10 (continued - 2)

Strains of S.lactis	Increase (over control) per 10 cc. filtrate amino N. mgms.	General rate of coagulation days	Strains of S.lactis	Increase (over control) per 10 cc. filtrate amino N. mgms.	General rate of coagulation days
L4	0.629	1	T3	0.000	4
M1	0.629	1	T4	0.027	4
M2	0.474	1	U1	0.399	1
M3	-0.080	2	U2	0.506	1
N1	0.474	1	U3	0.532	1
N2	0.458	1	U4	0.506	1
N3	0.674	1	V1	0.452	1
N4	0.539	1	V2	0.452	1
N5	0.134	2	V3	0.452	1
O1	0.421	1	V4	0.486	1
O2	0.342	1	V5	0.540	1
O3	-0.052	2	W1	0.000	1
O4	0.342	1	W2	0.486	1
P1	0.447	1	W3	0.513	1
P2	0.552	1	W4	0.513	1
P3	0.552	1	X1	0.540	1
P4	0.605	1	X2	0.513	1
Q1	0.500	1	X3	0.513	1
Q2	0.395	1	X4	0.594	1
Q3	0.342	1	Y1	0.513	1
R1	0.026	3	Y2	0.548	1
R2	-0.026	3	Y3	0.548	1
R3	0.026	3	Y4	-0.054	2
S1	0.472	1	Z1	0.384	1
S2	0.026	3	Z2	0.356	1
S3	0.026	3	Z3	0.027	4
T1	0.479	1	Z4	0.000	4
T2	0.532	1	Z5	0.000	4

TABLE 10 (continued - 3)

Strains of S.lactis	Increase (over control) per 10 cc. filtrate amino N. mgms.	General rate of coagulation days	Strains of S.lactis	Increase (over control) per 10 cc. filtrate amino N. mgms.	General rate of coagulation days
AA1	0.544	1	CC4	0.108	2
AA2	0.027	4	CC5	0.571	1
BB1	0.707	1	CC6	0.625	1
BB2	0.489	1	DD1	0.544	1
BB3	0.136	2	DD2	0.054	3
CC1	0.380	1	DD3	0.544	1
CC2	0.680	1	DD4	0.516	1
CC3	0.462	1	DD5	0.571	1
			DD6	0.489	1

and CC_4 showed the most rapid coagulation at room temperature but the rate was never as rapid as that of the cultures causing definite proteolysis. Repeated trials of the proteolytic activities and general rate of coagulation at room temperature with these exceptional cultures substantiated the results given in Table 10. Among these cultures G_4 coagulated milk most rapidly and for this reason was used in later studies carried out to determine the relationship of proteolysis by S.lactis to butter deterioration.

It is evident that the S.lactis cultures fall into two types, proteolytic and non-proteolytic; the first rapidly coagulates milk while the second shows a good deal of variation in the rate of coagulation but is never as rapid as the proteolytic type.

Influence of unfavorable conditions on proteolysis by S.lactis .

Certain of the S.lactis strains isolated were carried for a considerable time at room temperature by frequent transfers in sterile milk. Trials made during this period showed that the proteolytic activities were constant. In order to determine whether or not these activities would be influenced by unfavorable conditions, such as keeping the organisms in soil or chalked milk

for 4 months, a trial was carried out in which five strains of S.lactis were used. The cultures were placed in soil or chalked milk in Erlenmeyer flasks which were kept at room temperature and shaken daily for about 2 weeks and occasionally after that. The soil was very dry and much of the water from the chalked milk was evaporated at the end of 4 months. The cultures were recovered by transferring small portions of soil or chalked milk to sterile milk. Two of the cultures which were contaminated with mold were purified by plating on whey agar and picking the colonies. After recovery the cultures were made active by several transfers and were then inoculated to sterile milk with or without added $CaCO_3$. The incubation period was 7 days for milk without $CaCO_3$ and 10 days for milk with $CaCO_3$. Only amino nitrogen was determined.

The data are presented in Table 11. For comparison amino nitrogen values obtained before the cultures were added to soil or chalked milk are given. The results indicate clearly that culture 18 which showed definite proteolysis before subjecting to soil or chalked milk also brought about a definite proteolytic change in milk after recovery. On the other hand the cultures causing little or no proteolysis before being placed in soil or chalked milk also gave slight if any increases in amino nitrogen after recovery. The addition of $CaCO_3$ to the

TABLE 11

INFLUENCE OF UNFAVORABLE CONDITIONS ON PROTEOLYSIS BY S.LACTIS.

ROOM TEMPERATURE

Strains of S.lactis	Increase (over control) in amino N. per 10 cc. filtrate		
	Before putting in soil or chalked milk	After holding in soil or chalked milk for 4 months	
	Incubation 7 days without $CaCO_3$	Incubation 7 days without $CaCO_3$	Incubation 10 days with $CaCO_3$
	mgms.	mgms.	mgms.
18	0.578	0.385	1.332
G4	0.082	0.027	0.000
K5	0.027	0.055	-0.027
N5	0.027	0.000	0.054
58	0.000	0.055	0.082

milk increased the proteolysis.

From the data it is evident that keeping S.lactis cultures in soil or chalked milk for 4 months apparently did not affect their inherent proteolytic properties.

Influence of temperature on the proteolysis and rate of coagulation by S.lactis.

The data already reported in which a correlation between proteolysis and the rate of coagulation was noted, were secured at room temperature. In order to determine the influence of higher temperature (30°C. or 37°C.) on the proteolysis and the general rate of coagulation by S.lactis strains, a trial was carried out in which several cultures of known proteolytic activities were used. The amino nitrogen was determined on cultures which were incubated 10 days in sterile milk with added $CaCO_3$, while the general rate of coagulation was observed in sterile litmus milk tubes.

The data secured are given in Table 12. It is evident from the amino nitrogen values that the cultures (RB_2, RM_1 and 18) which showed a definite proteolytic action in milk at room temperature also caused proteolysis at 30°C. or 37°C. although in general the amino nitrogen values were lower with an increase in temperature. The

TABLE 1

INFLUENCE OF TEMPERATURE ON P
TION BY S. LACTIS.

Strains of S. lactis	History of cultures	Room temperature: General rate of coagulation (hours)	Room temperature: Increase (over control) in amino N. per 10 cc. filtrat (mgms.)
RB2	(Cultures carried)	24	1.055
SB2	(with frequent trans-)	48	0.000
RM1	(fers for about 1)	24	1.677
SM1	(month after isolation)	48	0.027
18	(Cultures carried)	24	1.332
G4	(with frequent trans-)	30	0.000
K5	(fers for a few days)	30	-0.027
N5	(after recovery from)	30	0.054
58	(chalked milk)	60	0.082
18	(Cultures carried with)	24	0.741
G4	(frequent transfers for)	30	-0.027
K5	(about 4 mo. after re-)	30	-0.027
58	(covery from soil or)	48	0.000
	(chalked milk)		
18	(Cultures carried with)	24	1.114
G4	(frequent transfers)	30	-0.027
K5	(for about 7 mo. after)	30	-0.082
N5	(recovery from soil)	30	0.000
58	(or chalked milk)	48	-0.054

E 12

N PROTEOLYSIS AND RATE OF COAGULA-

ver r rate	30°C. General rate of coagulation hours	30°C. Increase (over control) in amino N. per 10 cc. filtrate mgms.	37°C. General rate of coagulation hours	37°C. Increase (over control) in amino N. per 10 cc. filtrate mgms.
	12	0.865	12	0.784
	18	0.054	14	0.054
	12	1.810	12	0.865
	20	0.108	20	0.054
	12	1.142	12	0.788
	12	0.054	12	0.000
	12	0.027	12	0.054
	16	0.054	16	0.027
	36	0.027	-	0.054
	-	-	12	0.385
	-	-	12	0.027
	-	-	12	0.082
	-	-	60	0.000
	12	1.066	12	0.820
	12	0.027	12	0.027
	12	0.136	12	0.000
	16	0.027	16	0.054
	20	0.054	60	0.000

cultures showing little or no proteolysis at room temperature also failed to give a definite increase in amino nitrogen at the higher temperatures. The rate of coagulation of both the proteolyzers and the non-proteolyzers was influenced by the temperature in that it was more rapid at 30°C. or 37°C., than at room temperature. With the non-proteolyzers (SB_2, SM_1, G_4, K_5 and N_5) the rate of coagulation was so rapid that it almost reached that of the proteolyzers. Culture 58, was an exception in that its rate of coagulation was rapid at 30°C but slower at 37°C.

The results indicate that the proteolytic action of S.lactis organisms was a constant character and not susceptible to change with a change in temperature. However, the rate of coagulation of both the proteolytic and non-proteolytic strains was affected by the temperature and on the whole was more rapid at higher temperatures than at room temperature. The non-proteolytic strains were so rapid at higher temperatures that they coagulated milk about as rapidly as the proteolyzers. The general correlation between the proteolytic activities of S.lactis cultures and their rate of coagulation, therefore, is true only at room temperature.

Influence of repeated transfers at 37°C. on proteolysis by S.lactis.

In order to secure additional information concerning the influence of temperature on the proteolysis by S.lactis, two strains, one proteolytic and the other non-proteolytic, were carried with frequent transfers in sterile litmus milk tubes at room temperature and at 37°C., for a considerable time. The cultures were then inoculated into sterile milk with added $CaCO_3$. The milk inoculated with cultures carried at room temperature was incubated at room temperature while that inoculated with cultures carried at 37°C., was incubated at 37°C. After 10 days the soluble and amino nitrogen were determined.

Table 13 presents the results obtained. It is evident that culture 18 caused a definite proteolysis and culture G_4 no proteolysis, regardless of the incubation temperature used and the number of transfers. In the case of culture 18, the soluble and amino nitrogen values were slightly lower at 37°C., than at room temperature, which is in accordance with the data given in Table 12.

The results shown in Table 13 indicate clearly that the incubation temperature did not affect the inherent proteolytic property of either the proteolyzer or the non-proteolyzer.

TABLE 13

INFLUENCE OF REPEATED TRANSFERS AT 37°C. ON PROTEOLYSIS BY S.LACTIS

	Room temperature			37° C.		
	Transfers carried	Increase (over control) per 10 cc. filtrate		Transfers carried	Increase (over control) per 10 cc. filtrate	
		Soluble N. mgms.	Amino N. mgms.		Soluble N. mgms.	Amino N. mgms.
18 trial 1	12	3.248	1.144	15	2.576	0.735
18 trial 2	12	3.416	1.171	16	3.248	0.844
G4 trial 1	9	-0.224	-0.054	17	0.504	-0.054
G4 trial 2	10	-0.448	0.000	17	0.392	-0.027

Influence of varying numbers of transfers on proteolysis by S.lactis in pasteurized and sterilized milk.

In order to determine whether or not the proteolytic activities of S.lactis strains are influenced by repeated transfers in pasteurized or sterilized milk, a trial was carried out in which two cultures, one proteolytic and the other non-proteolytic, were employed. Each culture was carried through a series of 21 transfers in pasteurized milk and through another series of 21 transfers in sterilized milk, using each time 200 cc. of milk without $CaCO_3$ in a pint milk bottle. The cultures were incubated at room temperature and the amino nitrogen determined at suitable transfers, as a rule immediately after coagulation. The controls for pasteurized milk were kept cool by packing in ice until the inoculated milk was curdled.

The results secured are given in Table 14. The data clearly indicatethat strain 18 continued to definitely decompose the proteins of pasteurized or sterilized milk, while strain G_4 gave no proteolysis. In general the amino nitrogen values for culture 18 were lower than those obtained in previous trials, which may have been due to the fact that the cultures were analyzed immediately after coagulation.

TABLE 14

INFLUENCE OF VARYING NUMBERS OF TRANSFERS ON PROTEOLYSIS BY S.LACTIS IN PASTEURIZED AND STERILIZED MILK. ROOM TEMPERATURE.

Transfer No.	Treatment	Increase (over control in amino N. per 10 cc. filtrate. S.lactis 18 mgms.	S.lactis G4 mgms.
2	past.	0.268	0.000
	ster.	0.215	-0.026
3	past.	0.531	-0.079
	ster.	0.292	-0.026
4	past.	0.327	-0.054
	ster.	0.218	-0.082
6	past.	0.629	0.136
	ster.	0.328	0.081
9	past.	0.382	-0.027
	ster.	0.300	0.000
10	past.	0.218	-0.081
	ster.	0.218	-0.027
14	past.	0.243	0.027
	ster.	0.297	0.000
18	past.	0.379	0.054
	ster.	0.379	0.054
21	past.	0.243	0.000
	ster.	0.270	0.081

It is evident that the proteolytic activities of S.lactis cultures did not change during 21 transfers ar room temperature in either pasteurized or sterilized milk.

Results of Trials in Butter

Proteolysis by S.lactis in butter made in small lots.

Butter cultures are widely used in the manufacture of butter and large numbers of the S.lactis organisms contained in butter cultures are commonly carried into butter. In the trials reported it was noted that the S.lactis strains isolated from butter cultures were of the proteolytic type. This suggests that these S.lactis strains may proteolyze butter and in this way influence the flavor and aroma of fresh or stored butter. In order to secure information on this point, four trials were carried out. In each trial several lots of butter were made, from the same batch of cream, some after sterilization and some after pasteurization, using a non-proteolytic (C_4) and a proteolytic (18) strain of S.lactis. In some cases the cream was churned after inoculating the organisms and incubating at room temperature until the cream had a slightly sour odor, while in other cases 10 per cent of a milk culture was added to

the cream immediately before churning. In each trial control butter was made from uninoculated cream. The butter was packed in 1 pound paraffined paper cartons and stored at different temperatures for varying periods. The total, soluble and amino nitrogen were determined on fresh and stored butter. The values for soluble and amino nitrogen were adjusted to a standard total nitrogen basis. The butter was judged in a general way without attempting to score it.

The data obtained are given in Table 15. In general the soluble and amino nitrogen values for fresh butter made with culture G4 after either sterilization or pasteurization were higher than those for the control butter or the butter made with culture 18, but in the majority of cases the differences between the comparable lots were very small. With the stored butter the differences in the soluble and amino nitrogen values between the butter made with culture G4 and that made with culture 18 were not consistent. Sometimes higher values were obtained with culture G4 which is not in agreement with the non-proteolytic character of the organism. The comparisons between the fresh and the stored lots of butter from the same trial showed that in some cases proteolysis was considerably increased in the stored samples but the increases were not confined to the butter made with

TABLE 15

PROTEOLYSIS BY S.LACTIS IN BUTTER MADE IN SMALL LOTS.

Trial	Age and storage temp.	Method of culture additions	Butter made with	Nitrogen per 10 cc. filtrate: Sterile conditions Soluble mgms.	Sterile conditions Amino mgms.	Pasteurized conditions Soluble mgms.	Pasteurized conditions Amino mgms.	Remarks
I	Fresh	Inoculated to cream	Control	1.400	0.285	-	-	All had heated flavor due to sterilization; no difference.
			G4	1.159	0.349	-	-	
			18	-	0.372	-	-	
	7 weeks at 21°C.	Inoculated to cream	Control	-	-	-	-	Control and 18 were moldy; G4 was very slightly moldy
			G4	2.520	0.836	-	-	
			18	-	-	-	-	
	22 weeks at -23 to -18°C.	Inoculated to cream	Control	0.568	0.279	-	-	None showed deterioration, all had heated flavor; if there was any difference G4 had more pleasant flavor.
			G4	0.694	0.306	-	-	
			18	0.588	0.256	-	-	
II	Fresh	Inoculated to cream	Control	-	-	1.024	0.314	All had clean flavor; no difference.
			G4	-	-	1.221	0.348	
			18	-	-	0.913	0.259	
	6 weeks at 21°C.	Inoculated to cream	Control	-	-	2.254	0.608	All were slighly covered with mold spots which were scraped off before analyses.
			G4	-	-	1.106	0.395	
			18	-	-	1.330	0.413	
	22 weeks at -23 to -18°C.	Inoculated to cream	Control	-	-	0.700	0.273	All were very good; no evidence of deterioration; no difference
			G4	-	-	0.695	0.237	
			18	-	-	0.655	0.233	
	Fresh	Inoculated to cream	Control	0.826	0.311	0.916	0.316	Sterile samples had heated flavor; pasteurized samples were clean; inoculated sterile 18 had abnormal body
			G4	1.680	0.456	1.576	0.289	
			18	0.624	0.380	1.548	0.279	
		10 per cent added to cream	G4	1.635	0.313	2.100	0.494	
			18	1.876	0.391	1.520	0.430	
	9 weeks	Inoculated	Control	1.414	0.479	3.178	1.132	Inoculated G4 had
			G4	4.200	1.290	3.052	1.992	

		added to cream	G4	1.655	0.510	2.100	0.704	
			18	1.876	0.391	1.520	0.430	
	9 weeks at 21°C.		Control	1.414	0.479	3.178	1.132	
III		Inoculated to cream	G4	4.200	1.290	3.052	1.992	Inoculated G4 had cheesy flavor; all others had old stale flavor
			18	-	-	2.842	1.021	
		10 per cent added to cream	G4	4.116	1.380	4.200	1.240	
			18	2.786	1.127	3.150	0.960	
	21 weeks at -23 to -18°C.		Control	0.778	0.293	0.658	0.242	Inoculated sterile 18 was abnormal; no conspicuous deterioration; no difference between G4 and 18 samples
		Inoculated to cream	G4	0.756	0.332	0.834	0.255	
			18	0.748	0.162	0.815	0.319	
		10 per cent added to cream	G4	0.652	0.290	0.661	0.235	
			18	0.622	0.303	0.622	0.213	
	Fresh		Control	0.871	0.253	1.276	0.313	Sterile control was abnormal; all had clean flavor; no difference
		10 per cent added to cream	G4	1.181	0.386	1.512	0.361	
			18	1.156	0.530	1.381	0.396	
	4 weeks at 21°C.		Control	1.215	0.235	2.002	0.611	Sterile G4 was better than sterile 18; sterile samples had old stale flavor, pasteurized ones were moldy
IV		10 per cent added to cream	G4	1.526	0.493	1.209	0.453	
			18	2.016	0.640	2.082	0.666	
	17 weeks at -23 to -18°C.		Control	1.229	0.180	1.414	0.254	Sterile control was worse than both G4 and 18; no difference between G4 and 18 (sterile or pasteurized)
		10 per cent added to cream	G4	1.260	0.232	1.098	0.264	
			18	1.517	0.314	1.120	0.298	

culture 18 but also occurred with that made with culture G4 and even in the control samples. This suggests that there are factors other than the S.lactis organisms influencing the proteolysis in stored butter.

The different samples of fresh butter made from the same lot of cream showed no important differences in flavors and aromas except that all the sterilized cream butter had a pronounced heated flavor. As a rule this was slightly covered up in butter made from sterilized cream to which organisms were added. The butter stored at 21°C. for varying periods was in most trials very defective, but in general the lots in the same trial showed the same defect. The butter held at -23 to -18°C. on the whole, showed good keeping qualities; again no differences between the comparable lots of butter could be detected.

It is evident that under comparable conditions the differences in the soluble and amino nitrogen values between the butter prepared with a proteolytic strain of S.lactis and that prepared with a non-proteolytic strain did not indicate a proteolysis by S.lactis. The facts that the same general type of change was noticeable in stored butter made from a lot of cream, whether uninoculated or containing large numbers of S.lactis organisms of either a proteolytic or a non-proteolytic type and that no appreciable differences in flavors and aromas between

the comparable lots could be noted show that proteolysis by S.lactis did not influence the keeping quality of the butter.

Proteolysis by S.lactis in butter made under creamery conditions.

In order to obtain additional data on proteolysis in butter by S.lactis and its influence on the keeping quality, eight trials were carried out under practical creamery conditions. In each trial the cream was pasteurized at 62.8°C. (145°F.) for 30 minutes in one vat; after cooling to 21.1°C. (70°F.) it was divided into two equal portions and to each 10 per cent of a culture of either a non-proteolytic (G4) or a proteolytic (18) strain of S.lactis was added. The cream was then cooled down to 4.4°C.(40°F.) and, after holding from 2 to 3 hours, it was churned. No control churnings were made. In washing, salting (3 pounds of salt to each 100 pounds of butter fat) and working an attempt was made to keep the conditions identical with the two lots of butter. In all trials the butter was salted but, in the last two, unsalted butter from each lot was also secured. Portions of each lot of butter were packed in 12 oz. paraffined paper cartons and stored at 30°C., 21°C. or 3 to 4°C. Another portion from each churning was packed in a 10 pound tub and held in the refrigerator (-23 to -18°C.). The storage time

varied from 4 weeks to 15 weeks. After storage the tub butter was analyzed for moisture and salt. The total, soluble and amino nitrogen were determined on the fresh and the stored butter. The values for soluble and amino nitrogen were adjusted to a standard total nitrogen basis. The fresh butter and the butter stored at 30°C., 21°C., or 3 to 4°C. was judged in a general way without attempting to score it while that held at -23 to -18°C. was scored for flavor and aroma on the basis of 45 for perfect.

The values for moisture and salt are given in Table 16. It is evident that the percentages of moisture and salt are in agreement with those commonly found in commercial butter, except that in four instances the moisture is slightly higher than 16 per cent. The comparable lots of butter showed differences in moisture and salt contents but these were very small. In some cases the lots of butter made with culture G4 showed higher and in other cases lower values for moisture and salt than the butter made with culture 18. Since the variation in the composition of the butter is so small, it would not be expected to have any influence on the keeping quality of butter.

The data on the proteolytic changes in the butter are presented in Table 17. In general, the soluble and amino nitrogen values for the fresh butter show that the differences between the comparable lots of butter were negligible. The same thing was true with the stored butter

TABLE 16

MOISTURE AND SALT CONTENT OF TUB BUTTER HELD FOR 10 TO 15 WEEKS AT -23 TO -18°C.

Trial	Butter made with	Percent	
		Moisture	Salt
A	G4	14.50	1.95
	18	15.20	2.11
B	G4	15.90	2.03
	18	14.95	2.15
C	G4	15.65	2.07
	18	15.85	2.31
D	G4	15.45	2.30
	18	15.90	2.24
E	G4	15.90	2.43
	18	16.30	2.41
F	G4	15.90	2.20
	18	16.10	2.12
G	Unsal. G4	14.70	-
	Unsal. 18	14.85	-
	Sal. G4	15.65	1.70
	Sal. 18	15.85	1.80
H	Unsal. G4	15.40	-
	Unsal. 18	14.65	-
	Sal. G4	16.40	2.18
	Sal. 18	16.25	2.21

TABLE 17

PROTEOLYSIS BY S.LACTIS IN BUTTER MADE UNDER CREAMERY CONDITIONS.

Trial	Age and storage temperature	Butter made with	Nitrogen per 10 cc. filtrate: Soluble mgms.	Nitrogen per 10 cc. filtrate: Amino mgms.	Remarks
A	Fresh	G4	0.873	0.258	Both had clean flavor; no difference
		18	0.854	0.258	
	4 weeks at 21°C.	G4	1.064	0.240	Both were cheesy; no difference
		18	1.064	0.283	
	15 weeks at -23 to -18°C.	G4	0.957	0.219	Score 37.50
		18	0.938	0.218	Score 37.75
B	Fresh	G4	0.874	0.274	Both had slight advanced lactation flavor; no difference
		18	0.854	0.253	
	4 weeks at 21°C.	G4	1.100	0.233	Both were very cheesy; no difference
		18	0.952	0.214	
	8 weeks at 3 to 4°C.	G4	1.100	0.208	Both had old stale flavor; no difference
		18	0.882	0.237	
	15 weeks at -23 to -18°C.	G4	0.851	0.274	Score 37.50
		18	0.762	0.254	Score 37.75
C	Fresh	G4	0.882	0.392	Both had clean flavor; no difference
		18	1.008	0.346	
	4 weeks at at 21°C.	G4	1.038	0.274	Both were slightly cheesy; no difference
		18	1.052	0.295	
	8 weeks at 3 to 4°C.	G4	0.882	0.269	Both had old stale flavor; no difference
		18	0.882	0.219	
	14 weeks at -23 to -18°C.	G4	0.630	0.297	Score 37.50
		18	0.672	0.324	Score 37.00

TABLE 17 (continued - 2)

Trial	Age and storage temperature	Butter made with	Nitrogen per 10 cc. filtrate: Soluble mgms.	Nitrogen per 10 cc. filtrate: Amino mgms.	Remarks
D	Fresh	G4	0.770	0.265	Both suggested a slight advanced lactation flavor; no difference
		18	0.806	0.349	
	4 weeks at 21°C.	G4	0.846	0.266	G4 had slightly more aroma than 18; practically no difference
		18	0.834	0.310	
	8 weeks at 3 to 4°C.	G4	0.695	0.227	G4 had a slight developed flavor; slightly better than 18
		18	0.761	0.250	
	15 weeks at -23 to -18°C.	G4	0.702	0.248	Score 38.00
		18	0.649	0.285	Score 37.50
E	Fresh	G4	0.883	0.302	Both had a clean flavor; no difference
		18	0.932	0.275	
	6 weeks at 21°C.	G4	1.147	0.309	Both were cheesy; no difference
		18	1.113	0.309	
	8 weeks at 3 to 4°C.	G4	0.576	0.246	Both had old stale flavor; no difference
		18	0.864	0.219	
	14 weeks at -23 to -18°C.	G4	0.633	0.274	Score 37.00
		18	0.635	0.306	Score 38.00
F	Fresh	G4	0.801	0.338	Both had a clean flavor; no difference
		18	0.753	0.284	
	6 weeks at 21°C.	G4	1.033	0.318	Both were cheesy; no difference
		18	0.863	0.315	
	8 weeks at 3 to 4°C.	G4	0.625	0.222	Both had old stale flavor; no difference
		18	0.728	0.247	
	14 weeks at -23 to -18°C.	G4	0.674	0.261	Score 37.50
		18	0.588	0.244	Score 38.00

TABLE 17 (continued - 3)

Trial	Age and storage temperature	Butter made with	Nitrogen per 10 cc. filtrate: Soluble mgms.	Nitrogen per 10 cc. filtrate: Amino mgms.	Remarks
G	Fresh	Unsal. G4	1.571	0.300	Both had clean flavor; no difference
		Unsal. 18	1.584	0.327	
		Sal. G4	1.134	0.328	Both had clean flavor; no difference
		Sal. 18	1.162	0.326	
	2 weeks at 30°C.	Unsal. G4	1.097	0.299	There was a slight development of good butter flavor in both samples
		Unsal. 18	1.030	0.273	
		Sal. G4	1.120	0.218	18 was slightly better than G4;
		Sal. 18	0.945	0.266	G4 showed slightly cheesy flavor
	5 weeks at 21°C.	Unsal. G4	1.701	0.303	Both showed the same general
		Unsal. 18	1.461	0.275	type of change; no difference
		Sal. G4	0.806	0.255	Both were cheesy;
		Sal. 18	0.814	0.304	no difference
	10 weeks at -23 to -18°C.	Unsal. G4	0.950	0.311	Score 37.00
		Unsal. 18	1.063	0.346	Score 37.25
		Sal. G4	1.008	0.311	Score 37.00
		Sal. 18	1.042	0.279	Score 38.00
H	Fresh	Unsal. G4	1.568	0.287	Both had clean flavor;
		Unsal. 18	1.582	0.277	no difference
		Sal. G4	1.165	0.246	Both had clean flavor;
		Sal. 18	1.221	0.303	no difference
	2 weeks at 30°C.	Unsal. G4	1.165	0.271	18 was slightly better than G4;
		Unsal. 18	1.151	0.299	the difference was negligible
		Sal. G4	0.891	0.250	18 was slightly
		Sal. 18	0.921	0.272	better than G4
	5 weeks at 21°C.	Unsal. G4	1.554	0.346	G4 was slightly moldy;
		Unsal. 18	1.408	0.347	no difference in flavor
		Sal. G4	0.773	0.317	Both suggested slight rancid-
		Sal. 18	0.912	0.310	ity; no difference
	10 weeks at -23 to -18°C.	Unsal. G4	0.719	0.306	Score 37.00
		Unsal. 18	0.749	0.318	Score 37.25
		Sal. G4	0.702	0.283	Score 37.00
		Sal. 18	0.750	0.307	Score 37.25

although in a few instances there were appreciable but inconsistent differences. Sometimes the butter made with culture G4 showed higher values for soluble and amino nitrogen than the butter made with culture 18, which was unexpected from the non-proteolytic nature of the organism. Comparisons between the fresh and stored butter indicate that in some cases proteolysis occurred during storage while in others the values for soluble and amino nitrogen were lowered. This suggests that proteolysis in the butter may have been influenced by factors other than the S.lactis organisms.

The fresh butter from most of the trials showed clean flavors and aromas and no differences could be detected between the comparable lots. In two instances the fresh samples suggested a slight advanced lactation flavor but this defect was present in both the lots of butter. It is evident from the flavors and aromas of the butter stored at 30°C., 21°C., or 3 to 4°C. for varying periods that in general there was no difference between the butter made with culture G4 and that made with culture 18. The storage, as a rule, decreased the quality of the butter but the deterioration was evident in both of the comparable lots. In a few cases the butter made with culture G4 showed a slightly better quality while in others it showed more deterioration than the butter made with culture 18. This indicates that such differences could not be attributed

to proteolysis by S.lactis.

The scores of the tub butter held at -23 to -18°C. indicate that the quality of all the butter was very good. The differences in the scores of the comparable lots were very slight and not consistent, showing that proteolysis by S.lactis was not a factor influencing the keeping quality.

It is evident that, under comparable conditions, the differences in the soluble and amino nitrogen values and in the flavors and aromas between the butter prepared with a non-proteolytic strain of S.lactis and that prepared with a proteolytic strain did not indicate a proteolysis in butter. Apparently the ability of a S.lactis strain to proteolyze milk does not influence the keeping quality of butter containing large numbers of this organism.

DISCUSSION OF RESULTS

The results obtained indicate that proteolysis by S.lactis is evident even in freshly coagulated cultures and does not require the extended incubation periods or the addition of $CaCO_3$ employed by various investigators in their studies along this line. Since it can occur so rapidly, proteolysis by S.lactis may have already taken place in sour cream as received by dairy plants.

The data showing proteolysis by certain S.lactis strains confirm the findings of various investigators along this line in that the proteolysis is increased when the cultures are incubated for extended periods in milk containing added $CaCO_3$. The increased proteolysis in the presence of $CaCO_3$ may have been due to an increased growth of the organisms resulting from keeping down the acidity. Under practical conditions there may be factors which reduce the acidity of dairy products and thus tend to favor proteolysis by S.lactis. The increase in the amino nitrogen (as measured by the Van Slyke method) accompanying the increase in the soluble nitrogen indicates that at least some of the protein decomposition goes to the amino acid stage.

From the data it is evident that proteolysis by butter cultures likewise does not require extended incubation periods but may have occurred in freshly coagulated cultures grown in pasteurized milk without added $CaCO_3$. This fact is of considerable significance because butter cultures are regularly grown in pasteurized milk and are usually used soon after coagulation. It is evident that butter cultures as ordinarily employed in dairy plants have already undergone protein decomposition.

The proteolysis noted with the butter cultures is undoubtedly to be attributed to the S.lactis organisms

contained since, in the trials carried out, the associated organisms failed to decompose the protein. The fact that the addition of 1 per cent or 2 per cent sterile commercial lactic acid did not increase the soluble or amino nitrogen contents of milk, while a butter culture definitely decomposed the proteins of the same milk, suggests that the breaking down of milk proteins is not due to the lactic acid developed by S.lactis organism but to an enzyme produced by it. This enzyme apparently does not bring about as conspicuous and extended proteolysis as do some of the proteolytic enzymes produced by bacteria. It may be possible, however, to obtain butter cultures causing no proteolysis in milk because the data indicate that there are S.lactis organisms which do not break down the proteins of milk.

It is known that air supply has considerable influence on the acid development by S.lactis and butter cultures. The trials carried out, however, failed to show any relationship of air supply to the proteolytic activities of S.lactis strains or butter cultures. It was observed that the rate of coagulation of S.lactis strains was increased by the addition of peptone or alanine to milk but such additions did not change the inherent proteolytic activities of the cultures, the proteolytic strains still definitely decomposing the proteins of milk

while the non-proteolytic did not. However, the extent of proteolysis by S.lactis was somewhat influenced in that the addition of such materials slightly retarded the protein decomposition. The increase in the rate of coagulation of slow coagulating strains of S.lactis by the addition of peptone or alanine suggests that the organisms may be limited in their growth in normal milk through their inability to satisfy their nitrogen requirements. It is evident that, on the whole, proteolysis by S.lactis is a character which is not influenced by air supply or the addition of peptone or alanine.

The trials carried out on numerous S.lactis organisms isolated from various sources showed that the cultures fall into two types, one proteolytic and the other non-proteolytic. The proteolytic organisms are rapid coagulators while the non-proteolytic ones show quite a variation in their rate of coagulation at room temperature but are never such rapid coagulators as the proteolytic strains. The relationship noted is in agreement with that reported by Anderegg and Hammer. However, the general relationship between the proteolytic activities of S.lactis and the rate of coagulation does not hold at 30°C. or 37°C. because some of the non-proteolytic strains curdle milk as rapidly as the proteolytic strains at these temperatures. This probably explains in part the failure of

various investigators to note a correlation between proteolysis and rate of coagulation with S.lactis.

The proteolytic activity of a S.lactis strain appears to be a constant character because in the trials carried out with selected cultures it was noted that repeated transfers at the temperatures used or holding in soil or chalked milk for an extended period did not influence their inherent proteolyzing action on milk.

With butter there was no evidence of proteolysis by S.lactis since the soluble and amino nitrogen values for the butter made with a proteolytic strain were not consistently higher than those for the butter made with a non-proteolytic strain. This is in accordance with the work reported by several investigators who found that the large numbers of S.lactis organisms present in butter made with butter cultures rapidly die out on holding. It is evident that butter is a very poor medium for the growth of S.lactis because lactose, which is the milk constituent most readily attacked, is present only in very minute quantities while the comparatively resistant fat makes up from 80 to 82 per cent of butter.

The comparison between the fresh and the stored lots of butter made from the same batch of cream showed that, although in some instances the soluble and amino nitrogen were considerably increased during storage, the

increases were not confined to the lots made with a proteolytic strain but were also evident in the lots made with a non-proteolytic strain and even in the controls. This suggests that there are factors other than the S.lactis organisms that are responsible for the proteolysis brought about in the stored butter. Among these factors galactose and microorganisms other than S.lactis are possibilities.

It should be noted, however, that there are a number of difficulties in securing adequate information on the proteolysis in butter. The most important of these is the presence of only small quantities of protein material. Deviations in the soluble and amino nitrogen values that result from analytical errors accordingly give large variations in the final results. The difficulty of separating all the non-fatty material from the fat and of obtaining a clear filtrate from salted butter should also be recognized.

Some of the substances bringing about desirable or undesirable flavors and aromas in butter are in such small amounts that they cannot be determined by chemical means. The products of protein decomposition may be among these so that soluble and amino nitrogen determinations are not delicate enough to detect significant changes. The desirable or undesirable influence of these protein cleavage products should, however, be evident in the flavors and aromas of butter. In the work carried out this was

not the case because no appreciable differences in flavors and aromas between the comparable lots of stored butter could be noted. Moreover, the flavors and aromas of the butter made from a batch of cream showed the same general type of change during storage regardless of the proteolytic character of the S.lactis strain used. From the practical standpoint the differences in the flavors and aromas between the comparable lots of butter are more important than the differences in the soluble and amino nitrogen values. In fact the effect of S.lactis organisms on the flavors and aromas of butter rather than nitrogen distribution was the object of the investigation. Apparently the ability of S.lactis to proteolyze milk is of no significance in the deterioration of butter.

CONCLUSIONS

1. Certain cultures of S.lactis definitely proteolyzed milk, while others did not.

2. Proteolysis by S.lactis did not require extended incubation periods but was evident even in as short a time as 1 1/2 day without the addition of $CaCO_3$ to the milk.

3. Proteolysis was evident in freshly coagulated butter cultures grown in pasteurized or sterilized milk, showing that butter cultures as ordinarily employed in

dairy plants have already undergone protein decomposition.

4. Proteolysis was more pronounced with S.lactis cultures and butter cultures when $CaCO_3$ was added to the milk than when it was not.

5. The organisms associated with S.lactis in butter cultures, namely S. citrovorus and S. paracitrovorus, did not cause appreciable proteolysis when grown in milk, which suggests that the protein decomposition in butter cultures is primarily due to the S.lactis organisms.

6. Sterile lactic acid added to milk in quantities sufficient to bring the final acidities to 1 per cent or 2 per cent did not increase the amounts of soluble and amino nitrogen after a holding period at room temperature.

7. Air supply did not influence the proteolytic change brought about by S.lactis cultures or butter cultures.

8. The inherent proteolytic properties of S.lactis strains were not influenced by the addition of 0.1 per cent peptone or alanine to the milk, but the extent of proteolysis was somewhat affected in that the addition of these materials slightly retarded the protein decomposition.

9. The S.lactis cultures studied were of two types, proteolytic and non-proteolytic; the first rapidly

coagulated milk at room temperature while the second showed considerable variation in the rate of coagulation but was never as rapid as the proteolytic type at this temperature.

10. The general correlation between the proteolytic activity of S.lactis and the rate of coagulation did not hold at 30°C. or at 37°C.

11. Incubating at 30°C. or at 37°C., repeated transferring at room temperature or at 37°C. and holding in soil or chalked milk for 4 months without transferring did not influence the inherent proteolytic properties of S.lactis strains.

12. There were no consistent differences in the soluble and amino nitrogen values between the butter made with a proteolytic strain of S.lactis and that made with a non-proteolytic strain.

13. Proteolysis by S.lactis was not evident in butter stored at different temperatures for varying periods whether it was made in small lots or under creamery conditions.

14. There were no differences in the flavors and aromas between the butter made with a proteolytic strain of S.lactis and that made with a non-proteolytic strain.

15. In general, the quality of the butter was

decreased during storage but the deterioration was not due to proteolysis by S.lactis.

16. Apparently S.lactis strains causing proteolysis in milk are of no significance from the standpoint of the keeping quality of butter.

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