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THE DISTRIBUTION OF CERTAIN AMINO ACIDS IN THE SOLUBLE NITROGEN FRACTION OF MILK CULTURES OF *STREPTOCOCCUS LACTIS*

by

Max E. Morgan

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Dairy Bacteriology

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1948

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INTRODUCTION

Although Streptococcus lactis long has been recognized primarily as being saccharolytic because its most readily apparent action in milk is the production of lactic acid from the lactose present, there are numerous reports in the literature regarding its weak but definite proteolytic activity. These reports have indicated that S. lactis is able to increase the soluble nitrogen of milk cultures, as measured by chemical methods. This line of investigation has not revealed information regarding the manner in which the various amino acids are released from the milk proteins.

The advent of the microbiological method for quantitative determination of amino acids has provided a tool by which the distribution of these acids in many biological products may be determined. It was felt that the microbiological method could be applied to the problem of determining the distribution of amino acids in some of the soluble nitrogen fractions of milk cultures of S. lactis. This information would be a contribution to the understanding of proteolytic properties of S. lactis and also would help clarify the role played by this organism in the proteolytic processes associated with the ripening of cheese.

By determining the amino acid activity for ten different acids on both tungstic and lactic acid filtrates prepared from

15-day cultures of S. lactis and by subsequently determining leucine and phenylalanine activities of filtrates prepared after different periods of incubation, it was hoped considerable additional information regarding the proteolytic activity of S. lactis would be obtained.

REVIEW OF LITERATURE

Von Freudenreich (1897) demonstrated that oval coccus forms isolated from Emmenthaler cheese were capable of increasing the soluble nitrogen of sterile milk to which chalk had been added to neutralize the lactic acid formed by the organism. In more recent times similar experiments have been repeated by numerous workers using S. lactis, which probably is the same organism with which von Freudenreich was working. The results of these studies have been reviewed by Hammer and Babel (1943). It has been found that proteolysis by S. lactis occurs in both sterile milk and sterile milk to which calcium carbonate has been added. The change is not extensive enough to alter the appearance of milk but may be detected chemically, even in freshly coagulated milk. Proteolysis has been determined by various methods including the determination of increases in Sorensen formol titration values, Kjeldahl nitrogen values of various soluble fractions, and the Van Slyke amino nitrogen values. There appears to be considerable variation in the proteolytic power of different strains of S. lactis. This may or may not be correlated with the rate of acid production of the different strains.

During a study of the protein metabolism of several species of lactic acid bacteria, Braz and Allen (1939) found that after several weeks incubation, a culture of S. lactis,

as well as some of the *Lactobacillus* species, brought about a detectable increase in the protein content of milk cultures containing no added calcium carbonate. Their results indicated that this increase occurred at the expense of the proteose-peptone fraction and that changes in the amino acid fraction usually were small. In the presence of calcium carbonate, *S. lactis* brought about a considerable decrease in the protein and an increase in the proteose-peptone fraction. Provision of an additional quantity of amino acids in the form of added yeast extract usually resulted in a stimulation of the anabolic process, so that either smaller quantities of amino acids were produced or negative values were obtained after growth of the cultures.

Anderegg and Hammer (1929), in a study of a large number of *S. lactis* strains, found an increase in soluble nitrogen in some cases and a decrease in others. Addition of peptone tended to retard proteolysis or to increase negative values, while addition of calcium carbonate resulted in more extensive proteolysis.

To the best of this writer's knowledge there are no reports in the literature at the present time dealing with the distribution of amino acids in the various soluble nitrogen fractions resulting from the proteolytic action of *S. lactis* in milk cultures.

It would appear from the foregoing citations that the

nitrogen metabolism of S. lactis involves a simultaneous synthesis and degradation of proteins and their split products. There seems to be a rather delicate balance between these two processes which is easily upset by increasing the longevity of the cells or by supplying them with the basic units for protein synthesis.

Niven (1944) studied the nutrition of a number of strains of S. lactis in a chemically defined medium and found the amino acid requirements to be complex. A minimum of 14 amino acids was required to initiate prompt growth. The organisms had an absolute requirement for valine, leucine, isoleucine, methionine and arginine but was able to grow without tryptophan. Glutamine and asparagine were necessary for the initiation of growth.

Dunn and his associates (1945) described a method for the microbriological determination of tryptophan in unhydrolyzed protein-free filtrates of blood serum and plasma. The plasma or serum was deproteinized with tungstic acid and the resulting filtrates adjusted to pH 7.0. Assays on such filtrates were reported as "apparent free" tryptophan.

In a recent series of papers (Hier and Bergeim, 1945, 1946; Solomon et al, 1947; Woodson et al, 1948), "apparent free" values for a number of amino acids have been reported on deproteinized plasma and cerebrospinal fluid and on unhydrolyzed urine. In reporting "apparent free" amino acid values these workers have either overlooked the possible amino acid

activity of such amino acid residues in peptide forms or considered the activity of such forms present in their filtrates to be negligible.

Amino acid residues in some peptide combinations have been found to possess amino acid activity. Simmonds, Tatum and Fruton (1947a, 1947b) working with x-ray induced mutant strains of *Escherichia coli* which required phenylalanine, tyrosine or leucine, respectively, found that various simple peptide derivatives of these amino acids served as growth factors for the respective mutant forms. Krehl and Fruton (1948) have found that various peptide forms of L-leucine have growth promoting properties for *Lactobacillus arabinosus* and *Streptococcus faecalis*. They have shown that such activity depends on the position of the leucine residue in the peptide with respect to the other amino acid residues, the nature of the other amino acids and the time of incubation. L-leucyl-L-tyrosine gave up to 106 percent of the growth promoting activity for *S. faecalis* of that expected if all the leucine in the compound were available for growth. This compound had up to 102 percent leucine activity for *L. arabinosus*. Glycyl-L-leucine had up to 103 percent leucine activity for *L. arabinosus* and 72 percent for *S. faecalis*.

From these reports it may be concluded that the results of microbiological assays for amino acids on unhydrolyzed biological materials which are apt to contain the smaller

fractions of protein degradation can not accurately be reported as "apparent free" amino acid.

At the time the present study was undertaken there were numerous microbiological methods available for assay of individual amino acids. These involved the use of a variety of assay organisms, basal media and details of procedure. The literature on this subject is too extensive to justify review here. The uniform method reported by Stokes and his associates (Stokes *et al*, 1945; Gunness *et al*, 1946) made possible the assay of histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, tryptophan, phenylalanine and tyrosine with a single basal medium and two assay organisms, *Streptococcus faecalis* and *Lactobacillus delbrueckii* LD5.* The method was reported to be applicable to assay of natural products as well as purified proteins. It was found to be stable to non-specific stimulatory or inhibitory substances which might be introduced with samples. Recoveries of known amounts of amino acid added to proteins prior to hydrolysis were quantitative within ± 2 percent. Microbiological values obtained for purified and impure proteins were found to be in reasonably good agreement with those obtained by chemical methods.

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* *Lactobacillus casei*, according to Rogosa (1946).

EXPERIMENTAL

Methods

Isolation and identification of *S. lactis* cultures

Ten samples of sour cream were obtained from shipments received in the Butter Laboratory of the Department of Dairy Industry of Iowa State College. Each sample represented cream from a different producer. Appropriate dilutions of the samples were plated in tomato juice-peptonized milk agar and incubated at 21°C. for 48 hours. Typical colonies were picked from each of the platings and transferred to litmus milk. After 24 hours incubation at 30°C., two cultures which revealed typical litmus milk reactions for *S. lactis* were selected from each series of isolations and carried through two more transfers in litmus milk. At this time duplicate litmus milk cultures were prepared in screw-capped test tubes, incubated 24 hours, then frozen and held for future use at -10°C.

Microscopic examination of methylene blue stains of all the litmus milk cultures from which the frozen cultures were prepared revealed elongated cocci occurring in pairs and short chains. All produced acid, coagulation and reduction in litmus milk within 24 hours when incubated at 30°C. When transferred to litmus milk held at 10°C. each culture produced acid

or acid, coagulation and reduction within 15 days. No change was observed for 72 hours after the cultures were transferred to litmus milk held at 45°C. From these tests it was concluded that all of the cultures used were strains of S. lactis according to Sherman's classification (1937).

Preparation of skimmilk cultures

Two hundred ml. portions of fresh skimmilk were dispensed into pint screw-capped bottles. These then were weighed on a torsion balance to the nearest one-tenth gram and the weight recorded on a tag fastened around the neck of each bottle. Sterilization was accomplished by heating to 100°C. in flowing steam for 40 minutes on three successive days. The bottles of milk were allowed to stand at room temperature between heating periods. After the last heat treatment the bottles were allowed to cool to room temperature and then were placed in a 21°C. incubator over night before inoculation.

The frozen cultures which were to be used as inoculum were allowed to thaw at room temperature. A loopful of each was transferred to a tube of litmus milk and incubated for 24 hours at 21°C. They then were carried through three more transfers to insure maximum activity.

Duplicate 200 ml. quantities of skimmilk each were inoculated with four drops of a 24 hour litmus milk culture of each of the strains of S. lactis to be studied. These

large cultures were incubated along with duplicate sterile controls at 21°C. for the desired length of time.

Preparation of culture filtrates

All cultures and sterile controls were made up to their original weight by addition of distilled water. Each culture was agitated thoroughly by shaking and then mixing in a Waring Blendor. Replicate 26-g. samples of each culture and the controls (equivalent to 25 ml. of skimmilk of sp. gr. 1.033) were weighed into 150 ml. beakers.

Tungstic acid filtrates. Samples of each of the cultures and of the sterile controls were diluted with 20 ml. of distilled water and adjusted to pH 1.5-1.6 by addition of a measured quantity (usually 11 ml.) of 0.6N H_2SO_4. The mixture was agitated thoroughly during addition of the acid. After quantitative transfer to a 125 ml. bottle the mixture was brought to 68.7 ml. with water and 6.3 ml. of 10 percent sodium tungstate was added. The bottle was stoppered and shaken for three minutes. The material insoluble in tungstic acid then was removed by filtration through dry fluted Whatman #2 filter paper. The first portion of the filtrate collected was returned to the filter for a second filtration. All the filtrates prepared in this manner had a pH of 2.40-2.50. This is well below the limit of pH 2.85 found by Merrill (1924) to be the point at which complete precipitation of blood

proteins occurs with tungstic acid.

This method is essentially the same as that used by Hier (1945) in preparation of protein-free plasma filtrates for microbiological assay of amino acids. A preliminary investigation indicated that if the pH of the milk cultures or sterile controls were adjusted to 1.5-1.6 and an amount of sodium tungstate solution added to give a calculated final concentration of 0.7 percent tunstic acid, the resulting filtrates gave a minimum biuret reaction. These filtrates also were less toxic to the assay organisms than those obtained with concentrations of 0.8 and 1.0 percent tungstic acid.

Lactic acid filtrates. The second 26 g. sample of each culture was diluted to 75 ml. with water, heated in a boiling water bath with constant stirring for five minutes and cooled in running tap water. The coagulated protein was removed by filtration as described for the tungstic acid filtrates. Since the pH of all the cultures after dilution was 4.2, the sterile control samples were diluted to 60 ml., adjusted to pH 4.2 with a measured quantity of 10 percent lactic acid and then made to 75 ml. From this point the sterile control samples were handled in the same manner as the culture samples.

Preparation of unhydrolyzed filtrates for assay

Each milliliter of the above filtrates was equivalent to 0.33 ml. of the original skimmilk culture or control. A 50 ml. quantity of each filtrate was adjusted to pH 6.8 with

0.1N NaOH and made up to the desired dilution. The culture filtrates were diluted to 250 ml. while the sterile control filtrates were made to 100 ml.; thus the final dilutions were 1:15 and 1:6, respectively.

Preparation of hydrolyzed filtrates

Additional tungstic acid and lactic acid filtrates of the cultures and sterile control were prepared by the procedures already described. Fifty ml. of each of these was made 2N with H_2SO_4 by adding 6.4 ml. of 50 percent H_2SO_4 and bringing the volume to 60 ml. with water. The acidified filtrates were placed in 150 ml. beakers covered with larger beakers. Hydrolysis was accomplished by autoclaving at 15 pounds pressure for five hours.

Removal of excess sulfate from hydrolysates. While still hot, the hydrolysates were transferred to 125-ml. centrifuge tubes and treated with 18 g. of $Ba(OH)_2 \cdot 8H_2O$ in 25 ml. of boiling water. After thorough mixing, the precipitated $BaSO_4$ was removed by centrifugation. The supernatant was decanted from the centrifuge tubes and passed through Jena sintered glass crucibles to remove any precipitate which was not thrown down in the centrifuge tubes. The $BaSO_4$ precipitates were triturated with 50 ml. of boiling water, recentrifuged and the washings decanted. Washing of the precipitates was repeated a second time and the washings combined with the first supernatants. The hydrolyzed culture filtrates were

adjusted to pH 6.8 and brought to 250 ml., the final dilution being 1:15.

The hydrolyzed sterile control filtrates were adjusted to pH 3.5 and concentrated to 60 ml. under vacuum. The concentrated hydrolysates then were adjusted to pH 6.8 and diluted to 100 ml.; thus the final dilution was 1:6.

All filtrates, including the tungstic acid, lactic acid, tungstic acid hydrolyzed and lactic acid hydrolyzed, prepared from both the skimmilk cultures and the sterile controls were stored under toluene in screw-capped bottles in the refrigerator. Further dilutions of the filtrates were made when required at the time the assays were run.

Amino acid assay of filtrates

Assays for valine, leucine, isoleucine, threonine, arginine, methionine, histidine, tryptophan, tryosine, and phenylalanine were carried out on the *S. lactis* skimmilk culture filtrates and the sterile controls by means of the microbiological method described by Stokes and his associates (Stokes *et al.*, 1945; Gunness *et al.*, 1946).

Cultures of the assay organisms, *Lactobacillus delbrueckii* LD5 #9595 and *Streptococcus faecalis* #9790, employed in this procedure were obtained from the American Type Culture Collection.

The basal medium used for the assay was modified in that

hydroxyproline was omitted due to its unavailability at the time this work was in progress. This omission was not considered detrimental to the accuracy of the assays, since it has been shown by Dunn and co-workers (1947) that hydroxyproline is not required by or stimulatory for either of the organisms used in this procedure.

The standard curves and assays were incubated 65 hours at 37°C. After the tubes were removed from the incubator, growth of the assay organisms was arrested by steaming for 15 minutes at 100°C. When the cultures had been cooled to room temperature, the lactic acid produced by the assay organisms from the glucose in the medium in response to graded increments of amino acid was determined by titration with standard alkali.

An electrometric titration apparatus similar to that described by Rockland and Dunn (1947) was used. It consisted of a Model No. 7663-A1 Leeds and Northrup potentiometer with glass electrodes, which were fixed in place in a 75 mm. Pyrex funnel. The stem of the funnel had been removed and the funnel fused to the barrel of a heavy Pyrex stopcock. The stopcock was connected to a water aspirator to facilitate rapid discharge of titrated samples. A small capillary tube was conducted into the titration receptable reaching the apex of funnel and almost touching the stopcock plug. Nitrogen gas was bubbled into the assay culture to affect adequate stirring during titration. The standard alkali was directed into the

titration funnel from a 25 ml. burette.

The contents of each assay tube was emptied into the titration funnel and the tube rinsed with three 5 ml. portions of distilled water. The rinsings were emptied into the funnel and the solution titrated to an endpoint of pH 7.0.

Procedure for biuret test

A stable biuret reagent was prepared according to Hawk and Bergeim (1939) by adding one percent copper sulfate solution, dropwise, with constant stirring, to a 40 percent sodium hydroxide solution until the mixture assumed a deep blue color. The biuret test was carried out on all the unhydrolyzed filtrates before they were diluted for assay. Four drops of the reagent were added directly to one ml. portions of the filtrates. After agitation of the mixture for five minutes the development of a purplish-violet color was considered to indicate the presence of protein, whereas, a decided pink color indicated the presence of proteose or peptone. Very light pink reactions were believed to be due to the presence of peptides.

Results

Amino acid activity of unhydrolyzed tungstic and lactic acid filtrates of 15-day skimmilk culture of *S. lactis*

Amino acid assays of valine, leucine, isoleucine, threonine, arginine, methionine, histidine, tryptophan, tyrosine, and phenylalanine activity were made on unhydrolyzed tungstic acid and lactic acid filtrates of skimmilk cultures of five strains of *S. lactis* and a sterile skimmilk control, all of which had been incubated at 21° C for 15 days. The results of these assays, expressed as gammas of amino acid per milliliter of original culture, are shown in table 1. The assay values indicated are averages of closely agreeing figures obtained at from three to five contiguous assay levels.

The values indicated for both the tungstic and lactic acid filtrates of the sterile control probably are not entirely accurate. Preliminary trials, in which such filtrates were added to a complete basal medium, revealed that the filtrates could not be diluted less than 1:6 without markedly inhibiting the acid production of the assay organisms. For this reason, the sterile control filtrates as used in the assays could not be more concentrated than a 1:6 dilution. Since the amino acid activities of the filtrates were so low, nearly all of the titration values obtained at the various levels fell between

Table 1

Amino acid assays of tungstic and lactic ac
skimmilk cultures of five st

Amino acid	Amino acid equivalents							
	Sterile control		Culture 1 c		Culture 2 c		Culture 3	
	Tungstic	Lactic	Tungstic	Lactic	Tungstic	Lactic	Tungstic	La
Valine	7.5	8.7	113.4	123.3	130.7	147.3	99.9	11
Leucine	4.9	8.9	122.0	149.6	147.3	172.2	111.0	13
Isoleucine	<5.3	5.3	65.1	98.1	80.7	120.9	77.7	10
Threonine	3.0	6.4	74.4	78.2	71.4	77.9	60.8	6
Arginine	6.8	11.8	76.7	105.5	80.1	108.2	58.8	9
Methionine	3.5	4.8	32.7	37.7	29.7	37.2	24.0	3
Histidine	1.6	3.7	29.6	42.3	38.1	49.8	25.7	4
Tryptophan	2.5	3.7	14.0	20.0	14.5	17.7	12.0	1
Tyrosine	<3.7	3.7	79.1	122.6	30.0	115.1	54.5	9
Phenylalanine	2.8	5.1	93.0	174.6	101.4	172.7	82.8	16

* Expressed as γ/ml. of original culture.

** These are maximum ratios since the lactic acid filtrate values of the control were t
low to measure and the values used in calculation of the ratios undoubtedly are too

Table 1

: and lactic acid filtrates prepared from 15-day
ures of five strains of S. lactis

cid equivalents of filtrates*								Ratio of av. increase	
Culture 3 c		Culture 4 c		Culture 5 c		Av. increase			
Tungstic	Lactic	Tungstic	Lactic	Tungstic	Lactic	Tungstic	Lactic	Tungstic	:Lactic
99.9	110.1	103.4	117.3	95.4	109.2	101.0	112.7	1	: 1.12
111.0	139.4	110.0	138.8	110.7	135.6	115.3	138.2	1	: 1.20
77.7	106.1	69.5	105.0	46.4	81.5	>62.6	97.0	1	: 1.55**
60.8	67.5	60.8	68.1	50.9	56.4	60.7	63.2	1	: 1.04
58.8	96.2	55.2	81.9	65.0	98.0	60.4	86.2	1	: 1.43
24.0	33.5	24.3	32.7	18.3	28.1	22.3	29.0	1	: 1.30
25.7	40.2	25.8	36.0	28.4	40.8	27.9	38.1	1	: 1.37
12.0	15.2	11.1	14.6	12.5	15.8	10.3	13.0	1	: 1.26
54.5	98.7	49.4	100.8	44.0	80.0	>57.7	99.7	1	: 1.73**
82.8	162.9	79.2	173.7	75.0	136.8	83.5	158.0	1	: 1.89

control were too
btedly are too high.

the blank value and the second increment of amino acid of the various standard curves. As a general rule, assay results calculated from titration values falling in this portion of a standard curve are not as dependable as those calculated from values falling nearer the center of a standard curve.

Assays on tungstic acid filtrates

The assay values presented in table 1 for all ten of the amino acids on each of the five cultures reveal increases over those obtained on the sterile control. In comparing the average increases of the assay values of the cultures over the values for the control, the increases in leucine and valine were highest, followed by that for phenylalanine. The average increases for isoleucine, threonine, arginine, and tyrosine were approximately equal in magnitude. The increases in histidine, methionine and tryptophan were low, with tryptophan showing the lowest absolute increase.

Comparison between tungstic and lactic acid filtrates. All of the assay values determined on lactic acid filtrates were higher than the corresponding values on the tungstic acid filtrates (table 1). Comparison of the average increases of assay values of the lactic acid filtrates of the cultures over the corresponding control values reveals considerable deviation from the same comparison made on the tungstic acid filtrate values. The average increase was greatest with phenylalanine,

followed by those for leucine, valine, tyrosine, isoleucine, arginine, threonine, histidine, methionine and tryptophan in order of decreasing magnitude.

The ratios of the average increases of the tungstic acid and lactic acid filtrates indicate that the lactic acid filtrates had 89.3 percent more phenylalanine activity than the tungstic acid filtrates. The tyrosine activity of the lactic acid filtrates was approximately 70 percent greater, while that for threonine was only 4.2 percent greater. The ratios for the other acids fell between these limits.

Since the assays of the lactic acid filtrates were higher than the assays of the tungstic acid filtrates in all instances, it is doubtful if the assay values on these unhydrolyzed filtrates indicate free amino acid. When a qualitative biuret test was applied to the filtrates, the tungstic acid filtrate of the sterile control was negative, whereas the lactic acid filtrate gave a bright pink color, indicating the presence of proteose or peptone. A light pink color was obtained on the tungstic acid filtrates of all the cultures, and the lactic acid filtrates of all the cultures gave a bright pink color.

Apparently there is a certain degree of variability in the ability of the different strains of S. lactis to convert milk protein and protein intermediates into their component free amino acids or fractions thereof which possess amino acid activity. Both the tungstic and lactic acid filtrates of cultures 1c and 2c gave higher amino acid assay values, in

most cases, than those of 3c, 4c, or 5c.

Leucine and phenylalanine activity of unhydrolyzed and hydrolyzed tungstic and lactic acid filtrates of S. lactis skimmilk cultures after different periods of incubation

Assays for leucine and phenylalanine activity were made on both unhydrolyzed and hydrolyzed tungstic and lactic acid filtrates prepared from skimmilk[1] cultures of two strains of S. lactis which had been incubated for 1, 3, 7 and 14 days at 21°C. S. lactis strains 2c and 4c[2] were chosen for this experiment because of the apparent difference in their proteolytic activity as judged by the assay values obtained on the unhydrolyzed filtrates prepared from 15-day cultures.

Assays for leucine and phenylalanine were made because it was believed that they would provide the most information regarding the extent to which S. lactis liberates amino acids and protein degradation products containing residues of such amino acids from milk proteins. It has been shown that S. lactis definitely requires leucine in its metabolism, whereas, phenylalanine has only a stimulatory effect on its growth (Niven, 1944). In the previous experiment, the ratio of the

[1] Skimmilk used for these cultures was not from the same lot used in the previous experiment.

[2] Cultures of these strains were duplicates of the strains used previously and had been preserved in a frozen condition.

average increase in the phenylalanine assays of the unhydrolyzed lactic acid filtrates over that for the corresponding tungstic acid filtrates seemed to be exceedingly high. Because of the rather marked upward drift in the phenylalanine values obtained in the assays of the lactic acid filtrates as the levels of added diluted filtrate increased from 0.5 to 3.0 ml., it was suspected that the lactic acid filtrates contained factors stimulatory for Lactobacillus delbrueckii LD5 which were not present in the basal medium. It was believed that the present experiment might furnish additional information concerning this situation.

Leucine assays. The values for the leucine activity of unhydrolyzed and hydrolyzed tungstic and lactic acid filtrates prepared from skimmilk cultures of strain 2c and 4c after various periods of incubation are shown in table 2 and are plotted in figures 1 and 2. There was a very sharp increase in the leucine activity of the unhydrolyzed tungstic and lactic acid filtrates of the cultures of strain 2c during the first 3 days of incubation when compared to the low activity of the same filtrates prepared from the uninoculated control. The rate of increase was somewhat more rapid with the lactic acid filtrate, indicating that protein degradation products possessing leucine activity and insoluble in tungstic acid were being formed. The rate of increase in leucine activity for both filtrates fell off considerably after the third day. From the third to the fourteenth day the total leucine activities of the

Table 2

Leucine activity of unhydrolyzed and hydrolyzed tungstic and lactic acid filtrates of skimmilk cultures of two strains of *S. lactis* after different periods of incubation

Period of incubation (days)	Leucine equivalent as γ/ml. of culture			
	Tungstic acid filtrates		Lactic acid filtrates	
	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed
	Strain 2c			
0	3.0	15.8	7.1	154.5
1	48.0	66.0	57.0	189.9
3	89.0	109.0	119.0	265.5
7	102.5	146.4	137.7	252.5
14	135.0	159.0	177.0	298.5
	Strain 4c			
0	3.0	15.8	7.1	154.5
1	30.0	53.0	38.3	156.0
3	57.0	72.5	71.3	192.0
7	76.2	126.0	105.5	212.6
14	91.5	111.0	120.0	202.5

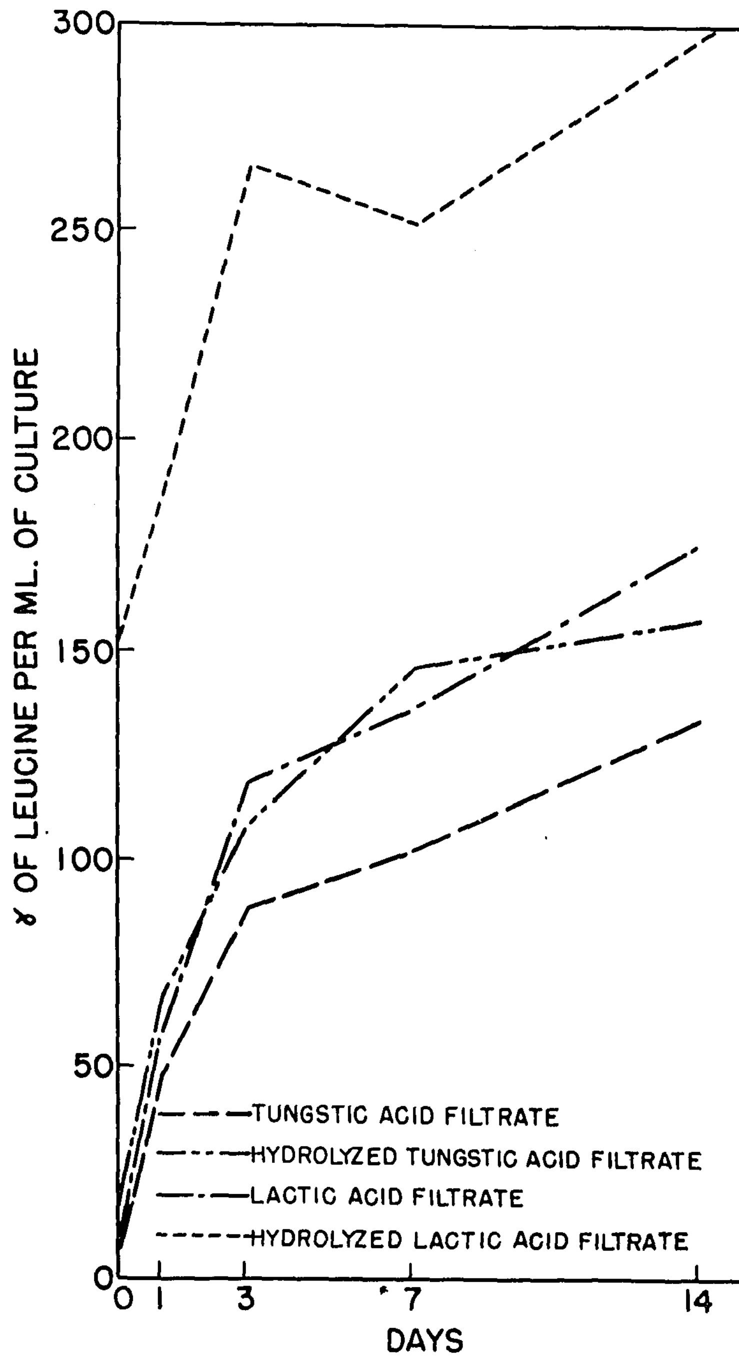

Fig. 1. Leucine activity of filtrates from cultures of S. lactis 2c.

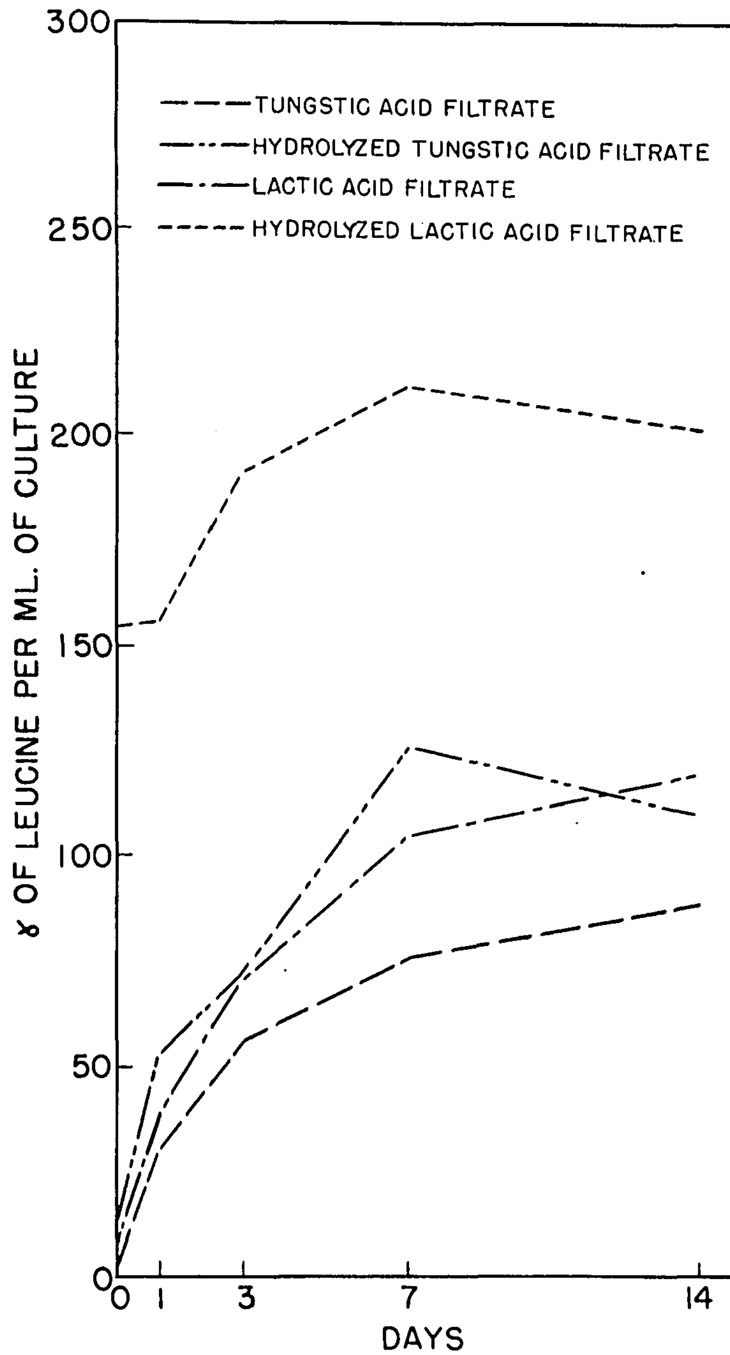

Fig. 2. Leucine activity of filtrates from cultures of *S. lactis* 4c.

two filtrates increased gradually at practically the same rate. The break in both curves which occurred on the third day of incubation probably corresponds to the end of the maximum stationary growth phase of the S. lactis cells, after which time the cells were dying at a rather rapid rate.

The high leucine activity of the hydrolyzed lactic acid filtrate of the sterile control as compared to the relatively low activity of the unhydrolyzed filtrate indicates that skimmilk sterilized by heating contains an intermediate fraction which could be polypeptide, peptone or proteose or a mixture of all three. From the increase in the assay value during the first three days incubation it would appear that some of the milk protein was hydrolyzed by the S. lactis cells. The reason for the apparent decrease in assay value on the seventh day is not readily apparent, unless it was due to an increase in the acid- and heat-coaguable protein in the form of S. lactis cells. This is quite unlikely, as the maximum number of S. lactis cells should have been reached at a point somewhere between the first and third day of incubation. The overall increase in leucine in the filtrates prepared on the third and fourteenth days indicates that the milk proteins still were being attacked by the proteolytic enzymes of S. lactis, even though the number of viable cells undoubtedly had decreased considerably during that time.

The data on the leucine assays on the hydrolyzed tungstic acid filtrates merely substantiates the previous contention,

namely, that the tungstic acid precipitation method employed will not remove all of the amino acid in peptide form from milk cultures in which partial protein hydrolysis has taken place.

Assay values on the unhydrolyzed tungstic and lactic acid filtrates prepared from cultures of strain 4c shown in table 2 and figure 2, when compared with the same values for strain 2c, indicated that strain 4c was somewhat less active in liberating leucine or hydrolytic products possessing leucine activity. On the seventh day the rates of increase in leucine activity of the tungstic and lactic acid filtrates became parallel. The hydrolyzed lactic acid filtrates from cultures of this strain revealed little or no increase in leucine activity during the first 24 hours incubation, but an appreciable increase did occur between the first and seventh day. This lag is further evidence that strain 4c was comparatively slow in its proteolytic activities. It is possible that the decrease from the seventh to the fourteenth day with this strain corresponds to the decrease between the third and seventh days with strain 2c. The overall increase from the first to the fourteenth day indicates a definite, though weak, proteolytic action by this strain.

Phenylalanine assays. The values presented in table 3 and plotted in figures 3 and 4 for the phenylalanine assays on the various filtrates reveal that the uninoculated sterile

Table 3

Phenylalanine activity of unhydrolyzed and hydrolyzed tungstic and lactic acid filtrates of skimmilk cultures of two strains of S. lactis after different periods of incubation

Period of incubation (days)	Phenylalanine equivalent as γ/ml. of culture			
	Tungstic acid filtrates		Lactic acid filtrates	
	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed
	Strain 2c			
0	0.9	3.2	3.4	3.2
1	25.5	32.6	59.3	57.0
3	54.0	56.3	111.8	75.0
7	70.5	36.5	145.2	148.2
14	80.1	78.9	174.0	187.5
	Strain 4c			
0	0.9	3.2	3.4	3.2
1	24.0	28.5	42.5	50.7
3	43.5	46.5	77.7	64.5
7	52.2	30.0	101.7	120.8
14	57.6	56.7	121.5	144.0

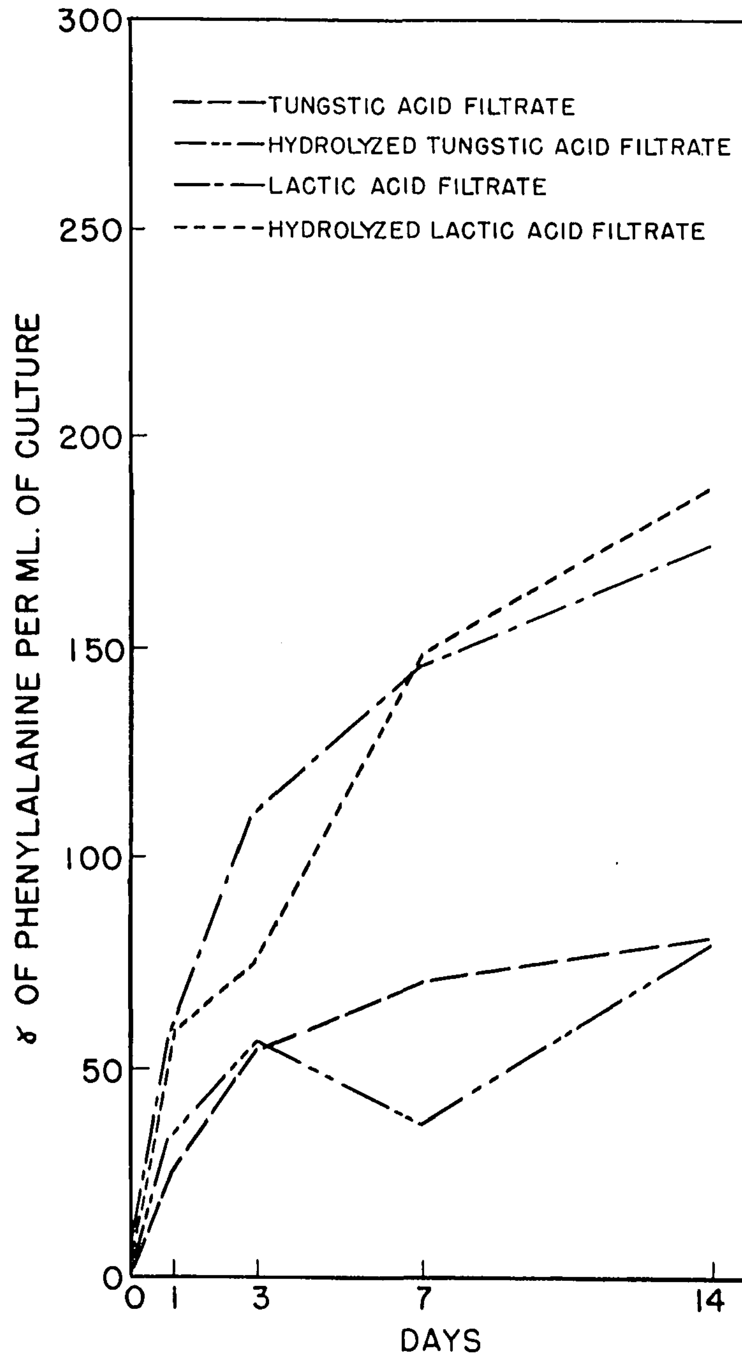

Fig. 3. Phenylalanine activity of filtrates from cultures of *S. lactis* 2c.

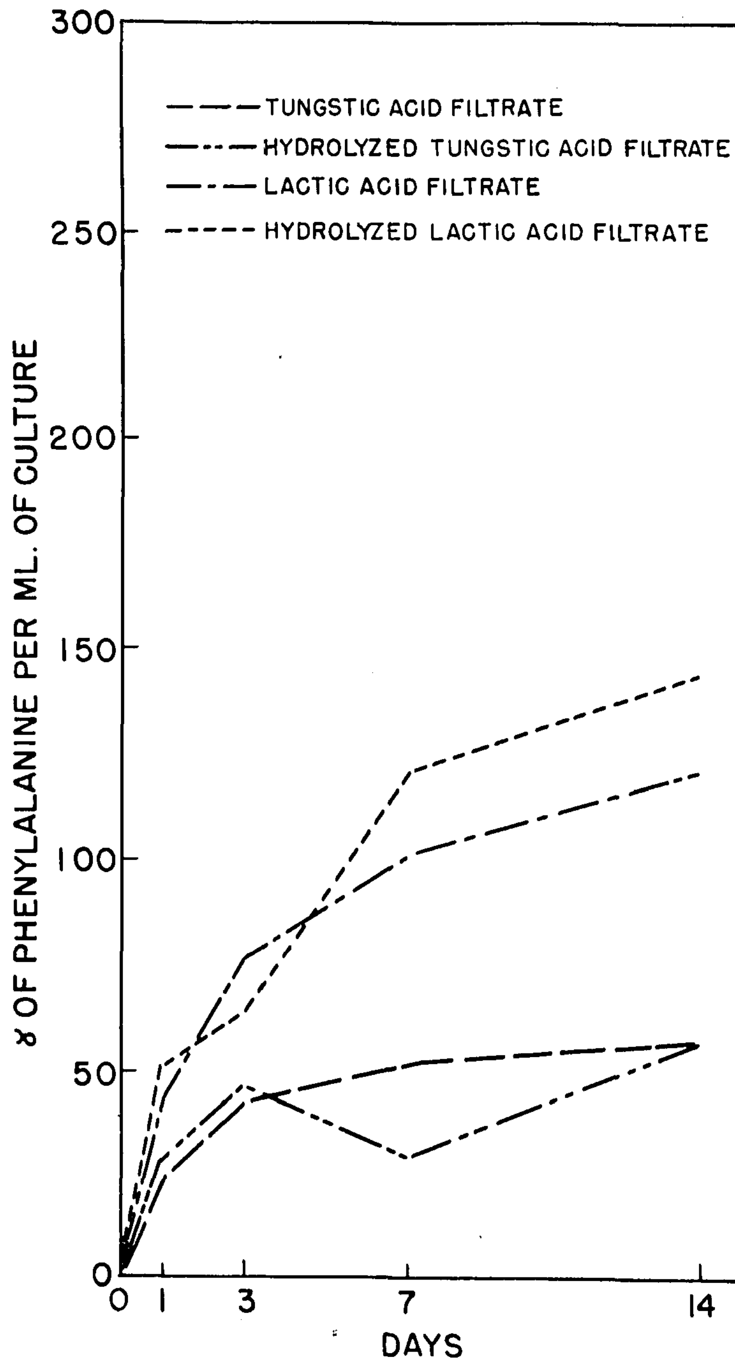

Fig. 4. Phenylalanine activity of filtrates from cultures of S. *lactis* 4e.

skimmilk contained very little free or combined active phenylalanine. The trends of the phenylalanine activities of the filtrates prepared from cultures of both strain 2c and 4c at the different incubation periods are nearly identical. The lower values obtained with strain 4c again indicate its lesser proteolytic activity. Comparison of the unhydrolyzed tungstic and lactic acid filtrates reveals that the latter had an average of more than twice the phenylalanine activity of the former throughout the 14-day incubation period. This would indicate that the lactic acid filtrates contain some intermediate fraction or fractions which have phenylalanine activity. This is further demonstrated by a comparison of the unhydrolyzed and hydrolyzed lactic acid filtrates. The unhydrolyzed filtrate activity approached that of the hydrolyzed filtrate throughout the entire incubation period and even surpassed it at the third day. The rather erratic variation in the assays of the hydrolyzed tungstic and lactic acid filtrate undoubtedly may be best explained on the basis of variation in the phenylalanine content of the protein degradation fragments produced after various periods of incubation. The marked depression of the hydrolyzed tungstic acid filtrate activity on the seventh day may be an indication that the *S. lactis* cells had converted some of the free phenylalanine into products having little or no phenylalanine activity for the assay organism. This occurred at a time when the activities

of the other filtrates were increasing due to the apparent high activity of some of the combined forms having phenylalanine activity.

Additional evidence is presented in table 4 which indicates that milk cultures of S. lactis contain a substance stimulatory for L. delbrueckii LD5 which is not acid- or heat-coaguable but is apparently inactivated by acid hydrolysis and may be precipitated with tungstic acid. The data presented are the actual assay values obtained at the various assay levels and include the values for all filtrates prepared after the 3, 7 and 14-day incubation periods on both strains 2c and 4c. The assay values for the unhydrolyzed and hydrolyzed tungstic acid filtrates show little, if any, drift with added increments of filtrate. The assay values for the hydrolyzed lactic acid filtrates show a slight tendency to decrease with increasing amounts of filtrate. The unhydrolyzed lactic acid filtrate values reveal a marked upward drift with each increment of added filtrate from the 0.5 ml. to the 3.0 ml. levels. This apparent stimulatory effect was never noted in the leucine assays of any of the filtrates.

Table 4

Phenylalanine assay values at various levels of added filtrates

Filtrate ml.	Phenylalanine equivalent as γ/ml. of diluted filtrate			
	Tungstic acid filtrate		Lactic acid filtrate	
	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed
	3-day culture of strain 2c			
0.5	3.6	3.0	5.8	9.2
1.0	3.6	3.9	6.5	10.8
1.5	3.6	3.7	6.8	9.6
2.0	3.6	5.7	7.7	9.8
3.0	3.6	3.7	8.8	9.4
	7-day culture of strain 2c			
0.5	5.0	4.0	8.0	10.6
1.0	4.8	4.2	8.6	10.2
1.5	4.7	5.0	9.3	9.8
2.0	4.6	5.0	9.8	9.9
3.0	4.7	5.2	11.0	9.6
	14-day culture of strain 2c			
1.0	5.0	5.0	9.9	12.6
1.5	5.2	5.2	11.1	12.9
2.0	5.5	5.3	11.8	12.6
2.5	5.5	5.4	12.7	12.4
3.0	5.5	5.4	12.6	11.8
	3-day culture of strain 4c			
0.5	2.8	3.0	4.4	4.2
1.0	2.9	3.1	4.8	4.4
1.5	2.9	3.1	4.9	4.4
2.0	2.9	3.1	5.1	4.4
3.0	2.8	3.1	5.9	4.0
	7-day culture of strain 4c			
0.5	3.6	4.0	6.0	8.8
1.0	3.6	3.6	6.1	8.1
1.5	3.6	4.0	6.4	8.1
2.0	3.4	4.2	6.8	8.1
3.0	3.3	4.4	7.8	7.9

(con'd next page)

Table 4 (con'd)

Filtrate ml.	Phenylalanine equivalent as γ/ml. of diluted filtrate			
	Tungstic acid filtrate		Lactic acid filtrate	
	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed
	14-day culture of strain 4c			
1.0	5.0	5.0	9.9	12.6
1.5	5.2	5.2	11.1	12.9
2.0	5.5	5.3	11.8	12.6
2.5	5.5	5.4	12.7	12.4
3.0	5.5	5.4	12.6	11.8

DISCUSSION

The evidence presented seems to indicate that unhydrolyzed filtrates prepared from *S. lactis* cultures contained intermediate products of protein degradation which possessed amino acid activity. For this reason the assay values on such filtrates cannot be assumed to represent only the free amino acid content of such filtrates. Since the lactic acid filtrates gave higher values than the tungstic acid filtrates for all amino acids for which tests were made, it must be concluded that the increase was due to intermediate fractions which were precipitable with tungstic acid but which were able to stimulate the growth of the test organisms. The tungstic acid filtrates undoubtedly contained a much smaller amount of compounds containing amino acid residues which gave amino acid activity. Also the biuret test on these filtrates indicated that compounds containing bound amino acids were present. Despite this limitation the amino acid activity values unquestionably are indicative of the degree to which free amino acids and the lower fractions containing these amino acids were liberated from milk proteins by the proteolytic action of *S. lactis*.

The results of the assays for the ten amino acids on the unhydrolyzed tungstic acid filtrates of the 15-day cultures of the five strains of *S. lactis* as compared to the assays on the

sterile control lend themselves to some rather interesting interpretation when tempered by the limitation recognized above. It was found that the five strains tested were able to liberate from milk proteins appreciable amounts of all ten amino acids in a form active in the assay procedure, i.e., as the free acid or an active combined form. It is interesting to note that there is a rather close relationship between the increase in the individual amino acid values as the result of the growth of S. lactis organisms and the relative amount of each amino acid present in milk proteins. The assay values for both milk and casein, as reported by Stokes et al (1945), indicate the following decreasing quantitative order of occurrence: leucine, valine, phenylalanine, isoleucine, tyrosine, threonine, arginine, histidine, methionine and tryptophan. The quantitative order of increase of values for these amino acids in the culture filtrates occurred in exactly the same order except that the value for tyrosine fell between arginine and histidine. This seems to indicate that the proteolytic enzymes of S. lactis liberate amino acids and their simpler compounds from milk proteins in amounts which are consistent with the occurrence of such amino acids in the proteins.

The assays on the lactic acid filtrates were included in this study in order to determine whether the presence of a greater amount of the intermediates of protein degradation in comparison to the amount present in the tungstic acid filtrates would have any effect on the determination. This experiment

was started before the reports indicating the amino acid activity of peptides of leucine, tyrosine and phenylalanine (Simmonds et al, 1947a, 1947b; Krehl and Fruton, 1948) came to the writer's attention. The marked increase in the amino acid activity of the lactic acid filtrates over that of the tungstic acid filtrates which occurred with all the assays except those for threonine is believed to be an expression of the amino acid activity of peptides. The apparent lack of difference between the threonine activities of the filtrates may indicate that the bound forms of threonine have little or no threonine activity or that threonine was not present in bound form in the protein degradation products remaining in the filtrate.

The increase in the leucine and phenylalanine assay values of the hydrolyzed lactic acid filtrates prepared from cultures of strains 2c and 4c after various period of incubation indicate that intermediates containing these amino acids were being split from the milk protein. The overall increase in these values from the first to the fourteenth day of incubation indicates that there must have been a decrease in total protein of the cultures. This is in direct opposition to the results of Braz and Allen (1939) which indicated a measurable increase in the total protein nitrogen of S. lactis milk cultures after six weeks incubation. If the total protein values of the present cultures were going to reveal any increases due to the synthesis of cell protein, it seems as though they would

have done so during the logarithmic and maximum stationary growth phases. The protein content probably would not increase during incubation extended beyond 14 days, as the number of viable cells would be decreasing during this period. Contrarily, the cells would be autolyzing, thereby increasing the total soluble nitrogen and releasing more proteolytic enzymes.

The rather large amount of leucine present in the soluble portion of the sterile control milk, as indicated by the assay value of the hydrolyzed lactic acid filtrate, indicates that sterilized milk contains some of the protein intermediates. It is quite likely that these intermediates constitute the proteose-peptone fraction which Rowland (1937) reported to be present in milk. He found that this fraction was insoluble in 8-10 percent trichloroacetic acid and amounted to 4 percent of the total nitrogen of normal milk.

The very low phenylalanine content of the soluble portion of the sterile control milk as indicated by the assays of the lactic acid filtrate reveals that the proteose-peptone and other non-protein nitrogen constituents contain little phenylalanine.

The lack of increase in the leucine content of the hydrolyzed lactic acid filtrate of the cultures of strain 4c during the first 24 hours incubation is considered to be evidence of low proteolytic activity. During this lag before the organism had

made any appreciable attack on the milk proteins, the leucine activity of the unhydrolyzed tungstic acid filtrate was increasing at a rapid rate. This may be considered as indicating that strains of S. lactis having low proteolytic activity derive amino acids for metabolism during their early phases of growth from the non-protein nitrogen fractions of milk. This was probably true for the active strain as well, but growth and metabolism were so much more rapid that any lag in proteolysis would be negligible.

The marked upward drift observed in all phenylalanine assays of the unhydrolyzed lactic acid filtrates undoubtedly is due to some substance present in these filtrates which has a non-specific stimulatory effect on the growth of the assay organism, L. delbrueckii LD5. There is no evidence in the present work which would reveal the nature of this substance other than that it is not acid- or heat-coagulable but is precipitated by tungstic acid and is destroyed by acid hydrolysis. It is quite probable that the phenylalanine activity of the unhydrolyzed lactic filtrates was high due to the presence of active phenylalanine peptides. This fact alone would not account for the stimulatory drift in assay values with increasing amounts of added filtrate.

All of the data in the present work appear to be directly applicable to further explanation of the role of S. lactis in the ripening of Cheddar cheese. It is quite probable that

an active _S. lactis_ cheese starter when added to cheese milk will increase the soluble nitrogen of the milk only slightly during the time allowed for proper acid development. Much of the soluble fraction produced at this time would be lost in the whey. Since a rather large proportion of the _S. lactis_ organisms are trapped in the curd and remain viable in the cheese in large numbers for several days and may be present in decreasing numbers up to three to four months, it seems quite plausible that these organisms would bring about some increase in the soluble nitrogen. From the present work it would appear that this change would include some breakdown of the cheese protein to the various intermediate and smaller fractions and small quantities of most of the amino acids present in milk protein. It is believed that even though the _S. lactis_ cells in the cheese die off during the early stages of ripening their proteolytic enzymes may continue their action after death and autolysis of the cells. The lactobacilli which are important in the later stages of cheese ripening are known to have some complex amino acid requirements. The amino acids and smaller fractions of protein degradation provided by the early action of the proteolytic enzymes of _S. lactis_ on the cheese protein apparently provide the necessary stimulus for development of the lactobacilli.

The stimulatory effect of the lactic acid filtrates of _S. lactis_ milk cultures on the growth of _L. delbrueckii_ LD5

which was noted in the phenylalanine assays may play a role in the stimulation of the growth of lactobacilli during cheese ripening. This may be at least a partial explanation of the effect which Hansen (1941) observed when he demonstrated that extracts of S. lactis have a stimulatory effect upon the growth of L. casei, as the assay organism employed for phenylalanine has been shown to be a strain of L. casei (Rogosa, 1946).

SUMMARY

Microbiological assays for valine, leucine, isoleucine, threonine, arginine, methianine, histidine, tryptophan, tyrosine and phenylalanine were performed on tungstic and lactic acid filtrates prepared from skimmilk cultures of five strains of S. lactis and a sterile control, all of which were incubated for 15 days at 21°C. The amino acid values for both filtrates of all strains revealed marked increases over the values for the filtrates from the uninoculated controls. The values for the lactic acid filtrates were considerably higher than those for the tungstic acid filtrates. This difference was believed to be due to the presence of a higher proportion of lower fractions of protein degradation products which possessed amino acid activity for the assay organisms employed. Evidence is presented which suggests that all of this lower fraction having possible amino acid activity is not precipitable with tungstic acid. It was concluded, therefore, that the assay values of the tungstic acid filtrates represent the free amino acid content of such filtrates plus the activity of some bound forms of amino acid.

The amino acid activity of the tungstic acid filtrates varied with the different amino acids, and it was noted that the proteolytic enzymes of S. lactis liberate free amino acids and small degradation fractions possessing amino acid activity

in the order of magnitude in which the amino acids occur in milk proteins.

Assays for leucine and phenylalanine activity were made on both unhydrolyzed and hydrolyzed tungstic and lactic acid filtrates prepared from cultures of two strains of S. lactis which had been incubated for 1, 3, 7 and 14 days at 21°C. The results of the assays on the unhydrolyzed filtrates indicated that active strains of S. lactis are able to affect a marked increase in the free amino acids and peptides possessing amino acid activity during the first 24 hours of incubation. The rate of increase declined after the third day of incubation and there was a slower but constant increase through the fourteenth day. This indicated that proteolytic enzymes of S. lactis continue their action after the culture has passed the maximum stationary growth phase and the number of viable cells is decreasing.

The simultaneous although not parallel increase in the assay values of the hydrolyzed lactic acid filtrates indicated that the total concentration of protein degradation products increased during the 14 day incubation period.

Evidence is presented which indicates the presence of a protein-intermediate fraction in sterilized milk. This fraction probably is the proteose-peptone fraction which has been detected chemically by previous workers. The present results indicate that it has a rather high leucine content

but contains very little phenylalanine.

Milk cultures of *S. lactis* contain a factor which is stimulatory to the growth of *L. delbrueckii* LD5, but is not coagulated by either acid or heat, is insoluble in tungstic acid and is destroyed by acid hydrolysis.

Implications concerning the role of the proteolytic activity of *S. lactis* in the Cheddar cheese ripening process have been discussed.

CONCLUSIONS

1. The microbiological assay procedures employed demonstrated that appreciable quantities of each of the ten amino acids studied and their compounds, probably small peptides, which were active in the assay procedure were freed from milk proteins by the growth of five strains of _Streptococcus lactis_ in milk cultures.

2. The quantities of these compounds liberated and detected are approximately proportional to the quantities of the individual amino acids found in milk proteins.

3. The increase of these forms of amino acid is most rapid during the period of rapid growth of the organism and continues at a slower rate to at least the fourteenth day of incubation.

4. Milk cultures of _Streptococcus lactis_ contain a factor which stimulates the growth of _Lactobacillus delbrueckii_ LD5. It is not coagulated by either acid or heat, is insoluble in tungstic acid and is destroyed by acid hydrolysis.

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