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DEGRADATION OF AMINO ACIDS BY LACTOBACILLUS CASEI IN RELATION TO FLAVOR DEVELOPMENT IN CHEDDAR CHEESE

by

Thorvald Kristoffersen

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Dean of Graduate College

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INTRODUCTION

The problems of flavor development and flavor constituents in Cheddar cheese and the reason(s) for the difference in flavor between Cheddar cheese made from raw and pasteurized milk are still largely unsolved. Lactobacillus casei, which increases in numbers during ripening, generally is considered to be responsible for the deeper changes in Cheddar cheese, but additions of L. casei to pasteurized milk for Cheddar cheese have not resulted in reproduction of the typical flavor of raw milk Cheddar cheese. This failure could indicate that lack of development of true flavor in such cheese either is not directly related to the action of L. casei or is not as much due to the reduction in the numbers of L. casei resulting from pasteurization as to a change in their metabolism. The sources from which L. casei derives energy for multiplication have not been established. The production of NH_3 by L. casei in chalk milk on prolonged standing and the increase in NH_3 nitrogen as Cheddar cheese matures indicate that amino acids could possibly be the source from which energy is derived, deamination being involved in the degradation.

Serine is an amino acid which particularly would lend itself as a source of energy for L. casei. The pathway for

deamination by L. casei, which is considered facultatively anaerobic, would be non-oxidative (hydrolytic). The resulting keto acid possibly should not be more than a three-carbon keto acid in order to be further metabolized by L. casei. These requirements are met by serine, which can be deaminated non-oxidatively and yields NH_3 and pyruvic acid upon deamination. Cysteine, which resembles serine in structure and can be deaminated in a similar manner, also could possibly be a source of energy.

Casein, the major protein constituent of cheese, would be the most important source of serine and cysteine. Serine makes up about 6 per cent of the amino acids in casein but a large part is present as phosphoserine. The presence of a phosphoric acid group has been shown to inhibit deamination of serine by other bacteria and greatly reduce hydrolysis by pancreatic enzymes. Presumably the inhibition by the phosphoric acid group also would apply to L. casei. This raises the question of the role of phosphatases, a group of milk enzymes which so far has not been considered important in the ripening of Cheddar cheese. The possibility was considered that the acid milk phosphatase with an optimum about pH 5.7 could be of importance. Preliminary studies showed pasteurization did not destroy this enzyme but resulted in a considerable reduction of activity.

The purpose of this study was to investigate the ability

of L. casei to deaminate amino acids. A few practical cheese making experiments were conducted to investigate the possible importance of degradation of amino acids by L. casei and the role of acid phosphatase activity in Cheddar cheese ripening, hoping some of the problems concerning flavor development and flavor constituents of Cheddar cheese could be elucidated.

REVIEW OF LITERATURE

Because several somewhat diverse subjects are reviewed in this section, the material has been divided into subsections, each of which covers closely related material.

Bacterial Deamination

The ability of gram positive dairy organisms, such as streptococci and lactobacilli, to deaminate amino acids is very restricted. Orla-Jensen (1919) reported the true lactic acid bacteria were incapable of splitting amino acids. Ayers et al. (1921) were the first to point out the value of NH_3 production by certain streptococci as an aid in differentiating species within this genus. Ayers et al. (1924) showed that arginine was the source from which NH_3 was released by certain streptococci. Peterson et al. (1928) tested 22 strains of lactic acid bacteria for proteolytic activity. Ammonia production was small in all cases and in some cases did not equal its consumption. Sherman (1937) and others have continued to use NH_3 production by streptococci as a means of classification, but this aspect will not be reviewed because it is not immediately related to the present problem.

Hills (1940) investigated the ability of streptococci

and staphylococci to deaminate amino acids and found only arginine was attacked to any extent. Aerobically other amino acids were not attacked by streptococci, but staphylococci were able to attack serine and threonine at very low rates. The breakdown of arginine gave rise to NH_3, CO_2 and ornithine and the name of arginine dihydrolase was suggested for the enzyme.

Rogosa et al. (1953) used the ability of certain lactobacilli to produce NH_3 in a medium containing tryptone and tryptose as a differential tool. Briggs (1953) classified lactobacilli on their ability to produce NH_3 from arginine. She used the medium described by Niven et al. (1942) for NH_3 production by streptococci.

Among gram negative bacteria, the ability to deaminate amino acids is more common. A complete coverage of the literature on this subject has not been attempted, as the number of different species of gram negative bacteria capable of deamination was considered immaterial to this study. Emphasis is put on literature dealing with the factors influencing deamination of serine and cysteine, especially by Escherichia coli which has been the favorite test organism.

Using the Warburg technique, Bernheim et al. (1935) showed that certain amino acids were deaminated and oxidized by resting cells of Bacillus proteus (Proteus vulgaris) at various rates. Serine required three atoms of oxygen for

complete deamination and oxidation. Only the natural isomers were oxidized, except for serine and alanine.

Stephenson and Gale (1937) demonstrated the ability of Bacterium (Escherichia) coli to deaminate glycine, DL-alanine and L-glutamic acid when grown aerobically. When the cells were grown anaerobically the ability was lost. Two per cent glucose in the growth medium also inhibited formation of deaminases.

Gale and Stephenson (1938) reported B. coli could deaminate DL-serine both aerobically and anaerobically. The activity of the serine deaminase was very high compared to other amino acid deaminases and varied with the age of the culture, with a maximum at 11 hr. The activity was increased by anaerobic growth conditions and, like other deaminases, was decreased 95 per cent or more by the presence of 2 per cent glucose in the growth medium. The loss of DL-serine deaminase activity with age appeared to be due to a loss from the cell by diffusion of some substance or substances acting as coenzyme. The loss of activity could be prevented by adding exudate from boiled bacteria, M/30,000 glutathionine or M/200 formate. All of these required the presence of M/100 phosphate, which alone brought about a slow recovery if decay had occurred anaerobically but not aerobically. This suggested the coenzyme may exist in reduced and oxidized and phosphorylated and non-phosphorylated forms, the reduced

phosphorylated form being relatively stable and non-diffusible.

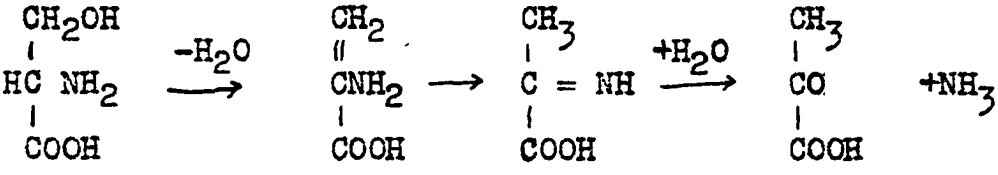
Gale (1938) found two aspartases in B. coli. One was unaffected by toluene and/or adenosine, while the other was completely inhibited by toluene treatment, required the presence of a coenzyme and was activated by adenosine in vitro.

Desnuelle and Fromageot (1939) reported on the anaerobic deamination of cysteine by B. coli. The breakdown was accompanied by increases in H_2S, NH_3 and pyruvic acid. Optimum was at pH 6.4.

Bacterium typhosum (Salmonella typhosa) was reported by Hills (1940) to deaminate serine and aspartate aerobically. Threonine and arginine also were deaminated to some extent. The formation of serine deaminase was inhibited 100 per cent and that of aspartase 70 per cent by 12.5 millimoles of glucose in the medium.

Chargaff and Sprinson (1943a) showed the inability of suspensions of resting B. coli to attack phosphatidyl serine isolated from beef brain, although the suspensions were very active in deamination of both D- and L-serine. Phosphatidyl serine contains the hydroxy amino acid in ester linkages via its hydroxyl group, which points out the importance of the free serine hydroxyl group for deamination. The same two investigators also found, in agreement with Gale and

Stephenson (1938) that B. coli would deaminate serine under aerobic as well as anaerobic conditions. These results suggested different pathways for deamination of serine and alanine. The following mechanism was suggested for deamination of serine:



pyruvic acid was demonstrated and isolated as a product of deamination of serine by B. coli.

In a subsequent article, Chargaff and Sprinson (1943b) reported on further studies showing that replacement of the hydroxyl hydrogen atom of serine always resulted in inhibition of deamination of serine by B. coli. One of the compounds tested was phosphoserine. Pyruvic and alpha-ketobutyric acids were identified as endproducts when serine and threonine, respectively, were deaminated by bacteria of different species.

Brinkley (1943) studied the nature of the enzyme involved in degradation of serine and cysteine. He reported that additions of glucose, serine or phosphoglyceric acid inhibited degradation of cysteine by extracts of E. coli or yeast. The inhibition was suggested to be of competitive nature as it disappeared on prolonged digestion. Because serine and cysteine give rise to pyruvic acid, and phosphoglyceric acid

to phosphopyruvic acid, the enzyme involved was believed to be enolase. The enzyme was found to be inhibited by fluoride in a manner identical with that reported for enolase. Dialyzed preparations lost their activity but this could be overcome by addition of Zn, Mg and Mn ions.

Lichstein and Christman (1948) investigated the role of biotin and adenylic acid in amino acid deaminases. Biotin deficiency of several bacterial species was obtained by holding the cells at pH 4 in phosphate buffer at 20-30° C. for 30-60 min. Such cells showed a markedly decreased ability to deaminate aspartate, serine and threonine but no difference in deaminase activity on alanine, phenylalanine, methionine and glutamic acid. The activity could be restored by addition of biotin or adenylic acid and by yeast extract, but not by a mixture of other members of the vitamin B complex. Biotin was suggested to be part of an essential coenzyme.

Wood and Gunsalus (1949) obtained serine and threonine deaminases from E. coli and partly purified them. The enzymes, which eventually were considered to be the same for deamination of both threonine and serine, were shown to require adenosine-5-phosphate and glutathione for activity. Yeast extract and other reducing agents, such as cysteine, sodium thioglycolate and ascorbic acid, were found ineffective. In view of Desnuelle and Fromageot's findings (1939) which suggested cysteine to be

deaminated in a manner similar to that for serine, cysteine was subjected to deamination by the partly purified enzyme preparation. Desulfarase activity could not be detected.

Metzler and Snell (1952) found the D-serine dehydrase of E. coli, unlike the L-serine dehydrase, was not activated by adenosine-5-phosphate but was by pyridoxal phosphate. No direct evidence was obtained that pyridoxal phosphate was a component of the enzyme deaminating L-serine and L-threonine, although its essential role in this process was considered likely.

Isolation of Phosphopeptones and Phosphoserine from Casein and the Effect of the Phosphoric Acid Group on Proteolytic Activity

Since Hammarsten (1883) first showed phosphorus to be a part of casein, not just an impurity, isolation of definite phosphorus-containing fractions of casein has been attempted. Rimington (1927a,b) and Posternak (1927) were the first to isolate such phosphorus-containing peptides. However, they did not agree on the composition of the peptides. Rimington isolated three moles of hydroxyglutamic acid, four moles of hydroxyaminobutyric acid, two moles of serine and three moles of phosphoric acid from his peptide, and postulated a structure with the phosphorus linkages between the three moles of

hydroxyglutamic acid and the first mole of hydroxyaminobutyric acid. Posternak's peptide consisted of three moles of glutamic acid, 0.6 moles of aspartic acid, three moles of isoleucine, three moles of serine and four moles of phosphoric acid. The phosphoric acid was thought to be bound to the hydroxyl group of serine.

Schmidt (1933) and Levene and Hill (1933) isolated from casein hydrolyzates a phosphorylated dipeptide made up of glutamic acid, serine and phosphoric acid. Lipmann (1933a) and Levene and Hill (1933) succeeded in establishing the position of the phosphorus by isolating serinephosphoric acid. Several other workers have reported isolation of this compound; Sorimati (1939) worked out a method to improve the yield.

Damodaran and Ramachandrau (1940), Lowndes et al. (1941) and Posternak and Pollaczek (1941a) isolated from casein phosphopeptones with from seven to ten amino acid residues and Rimington (1941) reexamined his original one. The peptides with from seven to nine residues yielded glutamic acid, isoleucine, serine and phosphoric acid; the decapeptides also yielded aspartic acid. Of particular interest is the work by Posternak and Pollaczek because it shows the effect the presence of phosphoric acid groups has upon the activity of proteolytic enzymes and also gives some information about the location of the phosphoserine in the peptides.

Casein was submitted to pancreatic digestion of relatively short duration and a phosphopeptide (I) made up of ten or eleven amino acids esterified with three phosphoric acid groups was obtained. This peptide was hydrolyzed only slowly by pancreatic enzymes, but hydrolysis was speeded up after enzymatic dephosphorylation. After a longer pancreatic digestion, a smaller peptone (II), made up of seven amino acids esterified with three phosphoric acid groups, was obtained. On hydrolysis peptone II yielded serine, glutamic acid and isoleucine; peptone I also yielded aspartic acid. The three phosphoric acid groups were attached to serine. Deamination by nitrous acid, after hydrolysis, yielded glyceric acid. After enzymatic dephosphorylation, the phosphopeptone could be oxidized by periodate, yielding formaldehyde and NH_3. This indicated the presence of a serinephosphoric acid residue at the end of the chain carrying the free amino group. Peptone II could be completely dephosphorylated by kidney phosphatase of swine, but peptone I lost only two of its three phosphoric acid groups under the same conditions. The difference was postulated to be due to the position of the phosphoric acid groups. In peptone I, two of these groups are at the end of the chain as serine-phosphate and one in the center of the chain. In peptone II, two of the groups are at the end of the chain but the third is located nearer one end, due to being part of a smaller

peptone. The phosphopeptones were resistant to aminopeptidases until enzymatic dephosphorylation had taken place.

Posternak and Pollaczek (1941b) further reported the inability of dipeptidases from hog intestines to act upon phosphoserylglutamic acid isolated from casein until dephosphorylation by phosphatase had taken place.

Monoester and Diester Linkages in Casein

Rimington and Kay (1926) postulated from their work on liberation of phosphorus from caseinogen and phosphopeptones by different phosphatases, that caseinogen and phosphopeptones are either a mixture of two or more substances containing organic phosphorus linked in different ways or that such linkages existed together in the same molecule.

Lipmann (1933b) found acid hydrolysis of phosphoprotein to be a monomolecular reaction. The reaction constant indicated that phosphorus is always bound to serine.

Sadamitsu (1937) investigated whether all the phosphorus in casein was in monoester form or if some part was in diester form. Liberation of phosphorus from casein hydrolyzate was demonstrated by phosphomonoesterases and phosphodiesterases, both alkaline and acid, from swine liver and rice bran, but

the investigator would not say for sure if all the phosphorus was in monoester form or if some had further combined to diester or pyrophosphoric acid.

Travia and Veronese (1940) were able to free all the phosphorus contained in casein with an alkaline phosphatase isolated from the spleen of oxen. They reached the conclusion that phosphoric acid probably is in the casein molecule in the monoester form.

Perlmann (1952a) demonstrated differences in action of prostate phosphatase (pH 5.6-6.6) on whole casein, alpha-casein and beta-casein. Phosphorus was readily released from alpha-casein but not from beta-casein and rather slowly from whole casein. In a later paper Perlmann (1954) showed the inability of prostate phosphatase to release phosphorus from beta-casein was due to the phosphorus being present in diester form. After having treated the beta-casein with a phosphodiesterase from snake venom at pH 7.2, the reaction was adjusted to pH 5.8 and prostate phosphatase added. Phosphorus was now readily liberated, indicating its presence in diester linkages in beta-casein. The results demonstrated the existance of cross-linkages of the phosphodiester type in casein, adding a new type to that of the disulfide bonds capable of linking peptide chains.

Milk Phosphatases

While phosphatases of living organisms have held the interest of investigators for many years and resulted in a multitude of publications, milk phosphatases have not held so much interest, at least from a physiological point of view. Probably because milk is an secreted product, the phosphatases present have been considered not to be of any further physiological importance.

Wilson and Hart (1932) were the first to determine alkaline phosphatase activity in cow's milk, but the work by Graham and Kay (1933) is considered classical in this field. These workers discovered an increase in the inorganic phosphate of cow's milk upon standing and showed this to be due to alkaline phosphatase activity. This enzyme was destroyed by heat and Kay and Graham (1933) were the first to use the destruction of the alkaline phosphatase as a criterion for proper pasteurization of milk.

Giri (1936) checked the phosphatase activity of human milk against sodium glycerophosphate at pH's ranging from 3.0 to 10.3. He found optima at pH's 9.2 and 5.1. The alkaline enzyme was activated some by Mg ions, while the acid was not.

Guittonneau et al. (1944) determined phosphatase activity on aqueous portions of butter, buttermilk, cream and milk.

Two phosphatases were found, one with an optimum at pH 4.2 and one at pH 7.6-7.8. Enzyme activity was weak in whole milk, absent in completely skimmed milk and present in proportion to the amount of fat in cream and in large amounts in buttermilk and butter serum. The acid phosphatase maintained its activity after heating to 73° C. for 50 min., while the alkaline enzyme lost its activity after heating to 63° C. for 20 min.

Vittu (1946) found three phosphatases present in human milk. One had its optimum at an alkaline pH and was activated by Mg ions. The two others were acid phosphatases, one with an optimum at pH 5.0-5.6 and indifferent to Mg ions and the second with an optimum at pH 3.8-4.2 and inhibited by Mg ions. All three preferred the beta form of sodium glycerophosphate.

Sjöström (1944) tested Alber's theory on dissociation and association of phosphatase on the alkaline phosphatase of cow's milk. Phosphatase activity at pH 9 could be restored in acidified milk samples if the pH of the original milk was not allowed to go below 4.0. The optimal reactivation of phosphatases in milk, whey and filtered sour milk was not obtained at pH 9 but at pH 6-7, the normal pH of milk.

A phosphatase with an optimum at pH 4.0 was found in cow's milk by Mullen (1950a). This enzyme hydrolyzed the alpha form of sodium glycerophosphate slightly faster than the beta form. Mg ions enhanced activity slightly and Mn

ions increased the rate of hydrolysis four times. Pasteurization reduced the activity of the enzyme 10-20 per cent.

Mullen (1950b) also found an acid phosphatase in mammary tissue of the cow. This enzyme had its optimum at pH 5.5-5.8 and hydrolyzed sodium beta-glycerophosphate two or three times as fast as sodium alpha-glycerophosphate. Addition of raw milk to the enzyme homogenate inhibited the phosphatase, while boiled milk did not. Using phenylphosphate, it was shown that there was about five times as much alkaline as acid phosphatase activity in the mammary gland of the cow.

Janecke (1950) could not find any acid phosphatase activity in milk.

Håkansson and Sjöström (1952) reported on an acid phosphatase in cow's milk. Using disodium phenylphosphate as substrate optimum activity was at pH 3.8-4.0. Enzyme activity was not destroyed by pasteurization, although some reduction took place. About 90° C. for 5 min. was required for complete destruction. The investigators did not think the reduction in activity due to pasteurization was due to destruction of alkaline phosphatase, since this phosphatase was shown by Sjöström to be inactivated at pH 4, but mentioned the possibility of the presence of two acid phosphatases.

Jacquet and Odette (1952) found three phosphatases in cow's milk. These enzymes had optima at pH 9.4, pH 5.5-5.8 and pH 3.5-4.0. Sodium beta-glycerophosphate was used as

substrate. The alkaline enzyme was about six times as powerful as the acid ones and was the only one to be completely inactivated by heat. The phosphatases with optima at pH's 9.4 and 3.5-4.0 were always present in milk, while the one with its optimum at pH 5.5-5.8 sometimes was missing.

Action of Milk Phosphatases on Casein

Whereas casein or smaller fractions of casein have been subjected to dephosphorylation by phosphatases from different sources, including the action of potato phosphatase on phosphoserine at pH 5.3 as shown by Perlmann (1952b), these substrates apparently never have been used to determine the activity of milk phosphatases. The only known results demonstrating release of phosphorus from casein by milk phosphatases are the original ones by Graham and Kay (1933). Their results showed a reduction of about 50 per cent in the ester phosphorus content of freshly drawn milk after standing 4 hr. at milking temperature. Prolonged holding did not result in further reduction. Cooling of the freshly drawn milk resulted in liberation of less ester phosphorus. The decreases depended on the extent of cooling; milk cooled rapidly after milking, and kept cold, did not show any decrease in the ester phosphorus content up to 24 hr. Chloroform did not inhibit the reaction. Addition of dilute

NaOH increased and addition of dilute lactic acid depressed the release of phosphorus. The phosphoric ester phosphorus of milk varied with the breed of cow, being lowest in the milk of Canadian Holstein-Friesians and highest in Jersey milk.

The Role of Lactobacilli in Cheddar Cheese

Hastings et al. (1912) studied the sequence of the common types of bacteria in Cheddar cheese. S. lactis dominated the flora in the early stages, with lactobacilli gradually taking over as the cheese matured.

Davis et al. (1934) added cultures of L. casei and L. plantarum to milk for Cheddar cheese. This resulted in an increase in non-protein nitrogen after 1 month. The difference became negligible after 5 months, due to increase of the lactobacilli naturally present in the control cheese. There was no apparent difference in flavor.

Davis (1935) studied over a 5 year period the lactic acid flora of Cheddar cheese made from certified milk. S. lactis and S. cremoris were found equally during the first month, after which the rods started to predominate. Streptobacterium (Lactobacillus) plantarum was present in the highest numbers from 1 to 5 months, after which the flora consisted entirely

of Sbm. casei.

Lane and Hammer (1935) added different strains of L. casei to pasteurized milk for Cheddar cheese and found this generally had a desirable effect on protein decomposition, flavor and uniformity of the resulting cheese. Some strains did not show added breakdown of protein. Some of the cheese developed a sour flavor after a while. This suggested to Lane and Hammer that development of sour flavor in Cheddar cheese was not necessarily due to the development of lactic acid from lactose, because lactose was used up in the early period of ripening. A general statement was made that there are other sources of lactic acid and also a number of possibilities in connection with the formation of acids other than lactic.

One of the more extensive investigations on lactobacilli in Cheddar cheese was carried out by Sherwood (1937) in New Zealand. He reported on results obtained by adding lactobacilli isolated from Cheddar cheese to pasteurized milk used to make Cheddar cheese. Both desirable and undesirable flavors resulted. Inoculation of cheese milk with mixtures of lactobacilli from mature cheese almost always led to fermented flavors. The causative strains of lactobacilli were isolated and shown to be of frequent occurrence in normal mature cheese, but under normal circumstances in insufficient numbers to spoil the flavor. The results of these

experiments were taken to indicate that the group of lactobacilli constitutes the most important flavor factor in normal Cheddar cheese.

Sherwood (1939a) isolated 720 strains of lactic acid bacteria from Cheddar cheese. Of the lactobacilli, Sbm. plantarum was found to appear most frequently and Sbm. casei less frequently. The flora of good quality cheeses was found to consist of one or two varieties of Sbm. plantarum associated with Sbm. casei. Addition of Sbm. casei to pasteurized milk for cheese in general appeared to be beneficial to the final product. Some strains of Sbm. plantarum were beneficial, but more produced serious defects such as bitter, unclean and "sulphide". Sherwood (1939b) also found that relatively small inocula of streptobacteria gave the best results. Large inocula imparted a sharpness to the cheese during the early stages of ripening and this sharpness often persisted when the cheese was mature.

Tittsler et al. (1946) reported cocci from the lactic starter predominated in young Cheddar cheese, regardless of quality or pasteurization of milk. After 1 month the flora of pasteurized milk cheese consisted almost entirely of enterococci, and that of raw milk cheese of lactobacilli, enterococci and a few diversified types.

Tittsler et al. (1948) found addition of L. casei to pasteurized milk used for Cheddar cheese resulted in increased

acidity and rate of softening of the body, but did not increase soluble nitrogen. It increased the development of flavor, but later caused an acid flavor and "short" body. Addition of L. plantarum seemed to show more promise.

Hunter (1950) sprayed Cheddar cheese curd with suspensions of L. casei, but the resulting increase in flavor was much smaller than could be desired. The size of the inoculum seemed to have little effect. Something seemed to be lacking in pasteurized milk curd to support good growth of L. casei. Spraying the curd with carbohydrates not fermentable by streptococci to supply the lactobacilli with a source of energy, did not result in increased flavor.

Amino Acids and Their Degradation Products as Indexes of Development of Characteristic Cheddar Cheese Flavor

Dahlberg and Kosikowsky (1948) reported a direct semi-logarithmic relationship between tyramine content and the intensity of flavor of Cheddar cheese. Tyramine was not considered the cheddar-flavor compound, but served as a means of measuring bacterial activity that accentuated flavor production. However, results by Hupfer et al. (1950) and by Dacre (1953a) did not support these findings. They found no

direct correlation between tyramine content and flavor development.

Barnicoat (1950) reported normal Cheddar cheese contained below 35 ppm. of sulphhydryl groups and H_2S was present after about 14 days. Lactic solutions failed to produce H_2S from casein, supporting the theory that lactobacilli are responsible for formation of H_2S (Sherwood, 1939a). Addition of H_2S to cheese gave a sharp flavor, which was not unpleasant.

Mulder (1952) stated that H_2S most likely is of importance for cheese flavor, as cheese always contains some of this substance. In the same article Mulder points out that NH_3 probably is not of great importance, especially in hard types of cheese.

Baker and Nelson (1949) found addition of individual amino acids to Cheddar cheese curd made from pasteurized milk had little effect upon the flavor of the resultant cheese, except when histidine and serine were used. Histidine resulted in a definitely undesirable flavor and serine resulted in a possible slight increase in flavor and a considerable increase in the number of lactobacilli.

Harper and Swanson (1949) conducted microbiological assays to measure the amounts of nine amino acids in five Cheddar cheeses of different flavor intensity. A relationship of quantity of amino acids to intensity of flavor was found.

Mixtures of the nine amino acids in proportions found in the cheese were added to a bland base and the product judged by a panel. The results showed that amino acids definitely contribute to the flavor of Cheddar cheese, but probably not to the aroma, because all judges commented on the fact that the samples were odorless.

Kosikowsky (1951b) investigated the liberation of free amino acids in Cheddar cheese made from both raw and pasteurized milk. Quantitative as well as qualitative differences were noted. In general, most of the amino acids appeared by 30 days and increased in concentration as the cheese matured, but with some exceptions. Some amino acids increased slowly and others seemed to decrease in intensity after ripening reached a certain point. Some qualitative differences were the presence of proline, tyramine and alpha-amino-butyric acid in raw milk cheese and asparagine in pasteurized milk cheese. These compounds never were observed in cheese made from pasteurized and raw milk, respectively. Quantitatively, glutamic acid, leucine-methionine, basic amino acids, valine and phenylalanine reached high concentrations in raw milk cheese, while in pasteurized milk cheese glutamic acid, basic amino acids, phenylalanine and asparagine reached high concentrations. On the other hand, glutamine, serine, threonine, proline and alpha-amino-butyric acids were found in low concentration in cheese made from raw milk, and aspartic acid,

alanine, glutamine, threonine and glycine developed in low concentration when the milk used was pasteurized.

Silverman and Kosikowsky (1953) reported watery mixtures of amino acids and amines, in concentrations corresponding to that of Cheddar cheese, had a cheesy flavor and, together with fatty acids, produced some of the characteristic Cheddar cheese flavor but not all.

Determination of free amino acids by paper chromatography of different varieties of cheese by Simonart and Mayandon (1952), Storgårds and Lindquist (1953a,b) and Kosikowsky and Dahlberg (1954) showed the great similarity both quantitatively and qualitatively in amino acid patterns of different types of cheese, which made it rather impossible to correlate the flavor of a specific cheese with a specific amino acid pattern.

Dacre (1953b) investigated the amino acid patterns of New Zealand Cheddar cheese and their possible contributions to flavor. There did not seem to be any correlation between appearance of a particular amino acid and Cheddar flavor. Individual amino acids, their amines or mixtures did not possess any aroma or taste which might suggest they contributed to the typical flavor of Cheddar cheese.

EXPERIMENTAL

Methods of Procedure

Description of sources and identification of lactobacilli

The test organism used in the majority of the experiments was selected from a group of four cultures, three obtained from the Bacteriology Department, University of Wisconsin, Madison, Wis. and one from the Dairy Bacteriology collection at Iowa State College. These four cultures had been identified as Lactobacillus casei and were not reidentified.

Other lactobacilli were isolated from raw and pasteurized milk Cheddar cheese, using the selective medium of Fabian et al. (1953). The samples of cheese made from raw milk were obtained from Lakeshire-Marty Co., Plymouth, Wis. and the samples made from pasteurized milk from the Dairy Industry Department, Iowa State College. These were from the collection of judging cheese, which included cheese from Wisconsin, Illinois, Oregon and Iowa.

The isolates were only identified as L. casei types. They were examined only for morphology, gram stain, acid production, limits of temperature for growth and colony type. Sugar fermentations were not determined.

Cell production

A V-8 juice[1] medium of the following composition was used for production of cells for deamination studies and for inoculation of milk for cheese, as well as for carrying stock cultures:

V-8 juice	100 ml.
Bacto tryptone	10 gm.
Yeast extract	10 gm.
NaCl	5 gm.
Starch	0.5 gm.
Tween 80[2]	1 gm.

Distilled water to 1 l.

Adjust pH to 7 and autoclave for 20 min. at 15 lb. pressure.

For deamination studies the necessary amount of medium, which depended on the amount of cells wanted, was inoculated with 0.1 per cent of a 24 hr. old culture and incubated at 32° C. for 16 hr., resulting in a final pH of 4.20-4.25. The cells were harvested on an International Centrifuge Size 1, Type SB, and washed three times in ice cold physio-

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[1]V-8 juice produced by Campbell Soup Co., Camden, N.J., is a mixture of eight vegetable juices.

[2]A polyoxyethylene derivative of sorbitan monooleate.

logical saline, using a total of approximately 100 times as much saline as cells.

For cheese 25 ml. of culture were added per 50 lb. of milk.

Determination of deaminase activity

The washed cells were suspended in sterile water using enough water so 0.5 ml. of suspension would contain 1-2 mg. of bacterial nitrogen. Immediately after suspension, 0.5 ml. was added to a test tube containing 1 ml. of 0.1 M phosphate buffer and 0.5 ml. of 0.05 M amino acid. The test tube was closed with a rubber stopper and incubated in a water bath at desired temperature for 2 hr., with allowances in time for the tube contents to reach the temperature of the water.

Amino acid solutions were made fresh for each experiment and were adjusted to the pH of the buffer. The amino acid-buffer mixture was steamed for 0.5 hr. and cooled just before addition of the cells. Controls without amino acids and without cells, as well as samples for check of pH before and after incubation, were carried along. The reaction was stopped by addition of 0.5 ml. of 25 per cent trichloroacetic acid.

Determination of ammonia

The contents of the reaction tubes were made to 15 ml.

with distilled water and the cells removed by centrifugation. Ten ml. of the supernatant were steam distilled to recover the NH_3, using the modifications in method and apparatus proposed by Van Devender et al. (1952) of the original method by Choi et al. (1946). However, it was found sufficient to use only 5 ml. of the 8 per cent $Mg(OH)_2$ and to collect only 9 ml. of distillate in a test tube containing 1 ml. of 0.01 N H_2SO_4.

NH_3 was determined by the method of Johnson (1941), as described in Manometric Techniques and Tissue Metabolism, Umbreit et al. (1951), using a Klett-Summerson photoelectric colorimeter with a 500 mu wave-length filter. A standard curve for NH_3 (Figure 1) was established by steam-distilling solutions of NH_4Cl containing various amounts of NH_3. The NH_4Cl used for preparation of the standard curve was dried previous to use.

The results are given as micrograms of NH_3 liberated per milligram of bacterial nitrogen per 2 hr. and are obtained by subtracting the endogenous reaction, no substrate added, from the metabolized.

Determination of pyruvic acid

Pyruvic acid was determined by the direct method of Friedman and Haugen (1943). A standard curve (Figure 2) was prepared by using dried sodium pyruvate. The measurements

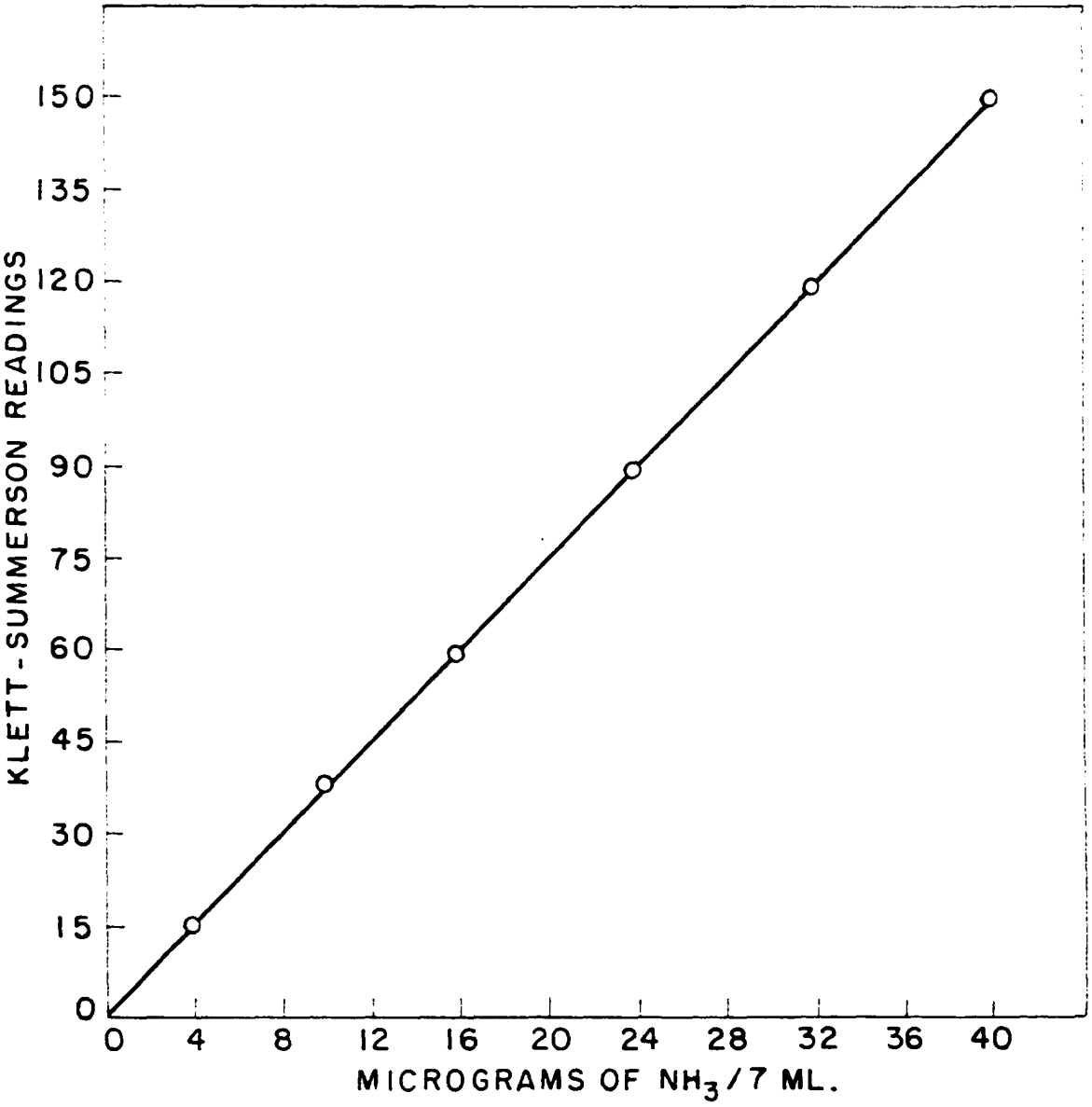

Figure 1. Standard curve for conversion of Klett-Summerson readings to micrograms of NH_3 per 7 ml.

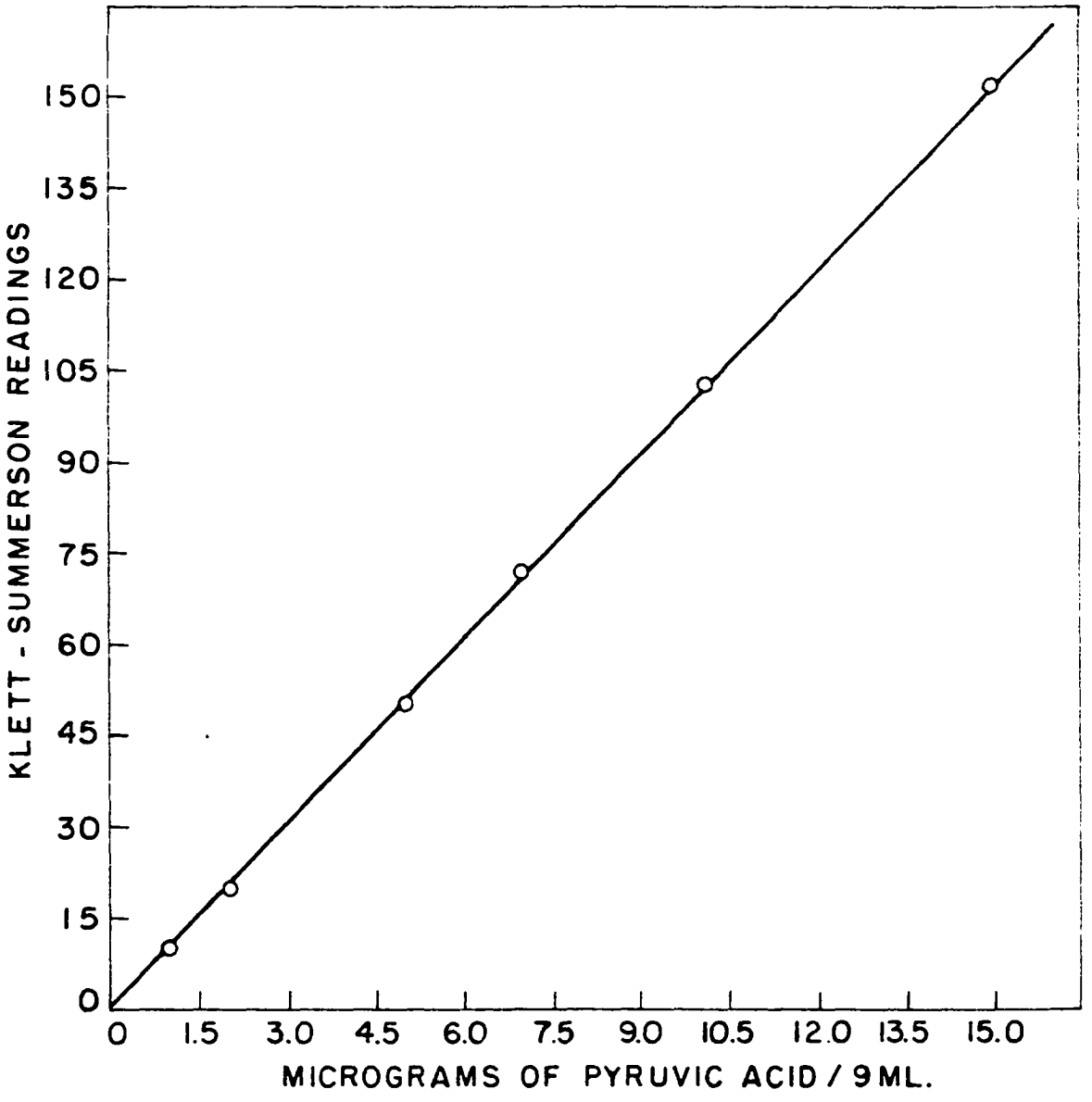

Figure 2. Standard curve for conversion of Klett-Summerson readings to micrograms of pyruvic acid per 9 ml.

on the Klett-Summerson photoelectric colorimeter were made with a 540 mμ wave-length filter.

Determination of lactic acid

Lactic acid was determined by the method of Barker and Summerson (1941). A standard curve (Figure 3) was prepared by using dried lithium lactate. The measurements on the Klett-Summerson photoelectric colorimeter were made with a 540 mμ wave-length filter.

Determination of pH

A Beckman model G glass electrode pH meter was used to determine pH.

Determination of nitrogen

The Kjeldahl method for total nitrogen, described by the Association of Official Agricultural Chemists (1945), was used for determination of bacterial nitrogen, total and soluble nitrogen of cheese and analysis of phosphoserine.

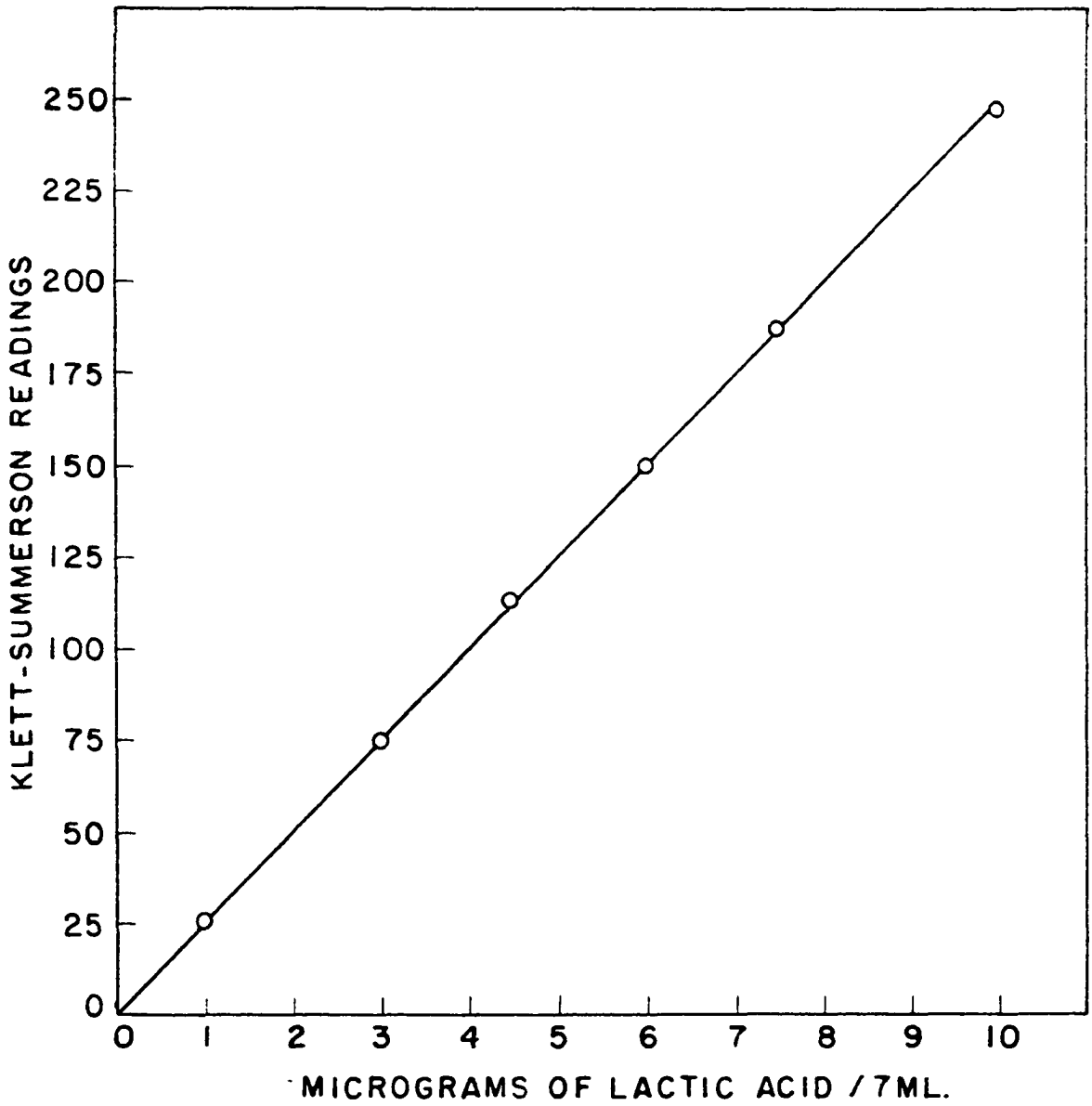

Figure 3. Standard curve for conversion of Klett-Summerson readings to micrograms of lactic acid per 7 ml.

Preparation of phosphoserine

Phosphoserine was prepared from casein (Nutritional Biochemicals Corporation) as the barium salt by the method of Sorimati (1939). Conversion to the sodium salt was accomplished by dissolving the barium salt in distilled water and adding dilute HCl to make the contents acid to congo red paper. Sufficient Na_2SO_4 was added to precipitate the barium. When converting to the sodium salt for analytical purposes, the contents were made to volume with distilled water and filtered through Whatman no. 42 filter paper. When the sodium phosphoserine solution was to be used for deamination experiments, the solution was adjusted to the desired pH with dilute NaOH before making to volume and filtering.

Determination of barium

Barium was determined gravimetrically as $BaSO_4$ by drying the filter paper and the $BaSO_4$ precipitate, obtained as mentioned above, at 140° F. under 20 inches of vacuum to constant weight on a Mojonnier tester.

Determination of phosphorus

Phosphorus was determined on the filtrate by the method

of Fiske and Subbarow (1925) as total phosphorus. A standard curve (Figure 4) was prepared by using dried K_2HPO_4. Measurements on the Klett-Summerson photoelectric colorimeter were made with the 645 mμ wave-length filter.

Preparation of whey globulin

Fifty lb. of raw whole milk was coagulated by rennet and the curd cut and allowed to settle. The whey was strained through four layers of cheese cloth; 12.6 l. were cooled to 3° C. and solid $(NH_4)_2SO_4$ added slowly with stirring to give half saturation. This degree of saturation was demonstrated by Palmer (1934) to precipitate the whey globulins. The precipitate was recovered by filtration and dialyzed against three shifts of distilled water at 3° C. to remove the $(NH_4)_2SO_4$. A period of 40 hr. was sufficient. Both filtration and dialysis were carried out at 3° C.

Preparation of potato phosphatase

Potato phosphatase was prepared by the method of Pfankuch (1936).

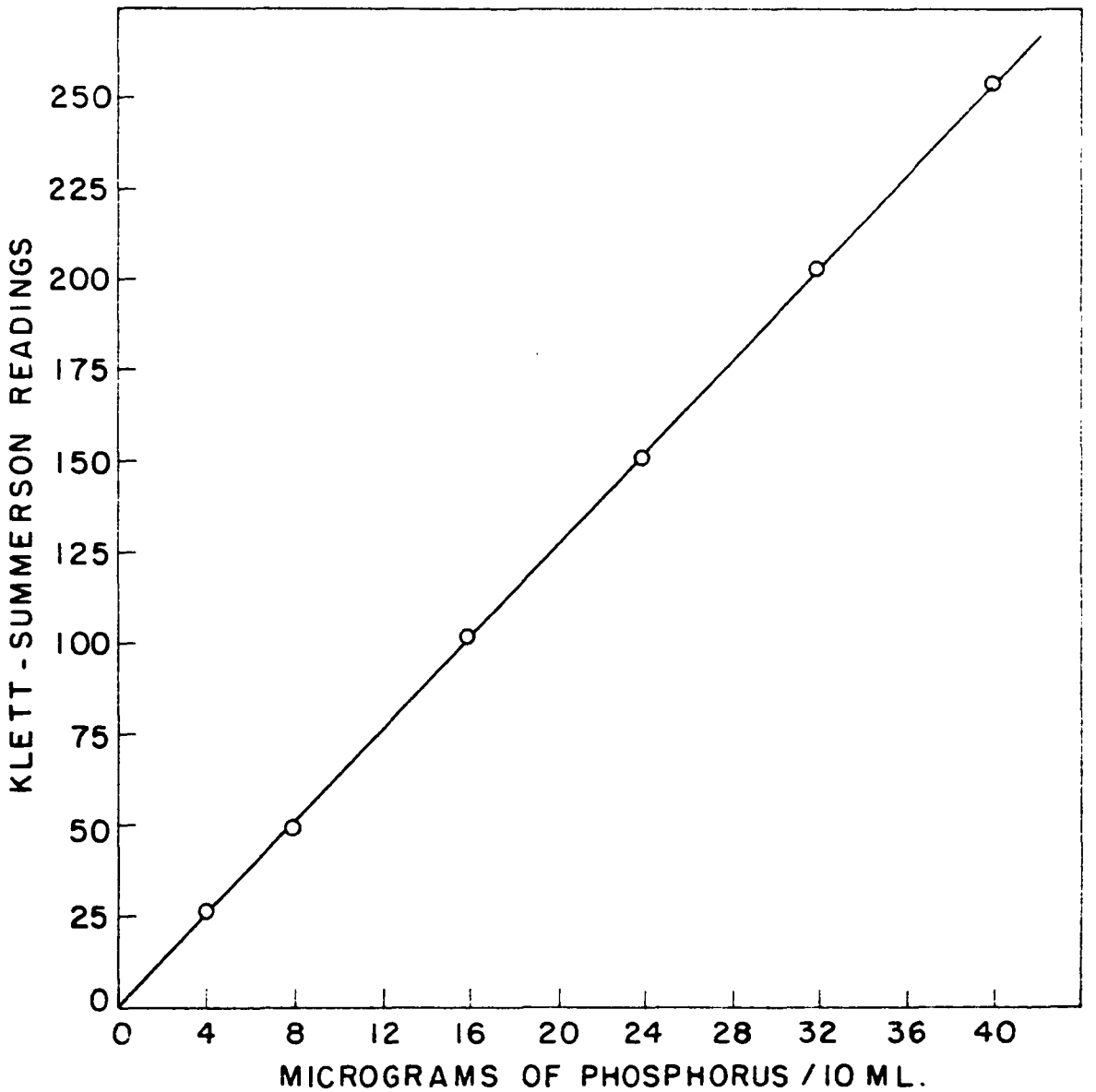

Figure 4. Standard curve for conversion of Klett-Summerson readings to micrograms of phosphorus per 10 ml.

Cheese making procedure

The cheese was made by the method outlined by Price (1944), except the milk was not ripened. Instead 2.5 times as much starter was used and rennet was added within 5 min. to overcome possible interference of bacteriophage, a procedure suggested by Whitehead and Harkness (1952). The milk was regular mixed milk from the Iowa State College creamery, testing 3.5-3.7 per cent fat and pasteurized at 161.5° F. for 15 sec. Each vat contained 155 or 212 lb. of milk, depending on the number of cheeses wanted. Approximately 50 lb. of milk made a 5 lb. wheel of cheese. The finished cheese was dried for 2 days at 50° F. before waxing. Curing was at 50° F. ±1° for 2 months and then at 42-45° F. for 4 months.

Scoring of the cheese

After 1, 3 and 6 months of curing the cheese was scored by two experienced judges. The system of the American Dairy Science Association, as described by Trout *et al.* (1942), was followed.

The intensity of characteristic Cheddar cheese flavor did not always correspond to the highest flavor score because of the presence of undesirable flavors. Therefore the cheese at 3 and 6 months were given from one to three plusses in addi-

tion to the numerical scores according to the increasing intensity of characteristic Cheddar cheese flavor.

Bacterial examination of cheese

The method proposed by the American Public Health Association (1953) was used for obtaining the sample of cheese. One gm. of cheese was then obtained aseptically from several places in the interior of the cheese plug and ground with 9.5 ml. of 2 per cent sodium citrate at 40° C. in a sterile mortar to give a 10^{-1} dilution. Further dilution was carried out in the regular manner described by the APHA.

As the change in numbers of lactobacilli was of prime interest, the previously described selective medium of Fabian et al. (1953) was used. A preliminary check showed that the S. lactis culture used as cheese starter, which normally would make up the predominant flora in cheese in the early stages, was unable to grow on this medium. As a further precaution against growth of streptococci the pH of the medium was lowered to pH 5.3 and the plates were incubated at 37° C., rather than at 32° C., for 48-60 hr.

Nitroprusside test for sulphhydryl groups in cheese

The method suggested by Barnicoat (1950) was used for a

quantitative estimation of -SH groups in the cheese.

Lead acetate paper test for hydrogen sulfide in cheese

This test was used as a qualitative and comparative test for presence of H_2S in the cheese. Some freshly cut cheese was quickly ground in a small meat grinder. A 25 gm. sample was put into a 500 ml. erlenmeyer flask with a 1.25 inch opening, together with 100 ml. of distilled water and 4 ml. of 25 per cent (by volume) H_2SO_4. A 4.5 X 4.5 inch piece of filter paper was held over the opening of the flask with a rubber band and moistened with a 10 per cent solution of lead acetate. The contents of the flask were slowly brought to boiling which was continued for 5 min. The filter paper was kept moist with lead acetate solution at all times. The intensity of the black area was recorded by one to four plusses at each of the three examination periods.

Determination of phosphatase activity at pH 5.4 in milk, whey, whey globulins and potato phosphatase solution

The method used was an adaptation of Sanders and Sager's method (1946). Acetate, 2-amino-2-methyl-1,3-propanediol (AMP) buffer (Tsuboi and Hudson, 1953) was used instead of borate buffer. The following is an outline of the method

as employed:

1. Take 10 ml. 0.1 M AMP buffer of pH 5.4 with disodium phenylphosphate added at the rate of 1.0 gm. per liter.
2. Add 1 ml. of the material to be tested, previously adjusted to pH 5.4 with 1 N HCl, and 1 drop of toluene.
3. Incubate in water bath at 37° C. for 1 hr. with allowance in time for the tube content to reach the temperature of the bath. Invert the tubes five or six times during this period.
4. Add 1 ml. zinc-copper precipitant to stop the reaction.
5. Adjust to pH 9.0-9.1 with 1 N NaOH and make to 15 ml. with distilled water.
6. Filter through Whatman no. 42 filter paper.
7. Pipette 5 ml. of the filtrate into a 40 ml. centrifuge tube.
8. Add 5 ml. color development buffer and four drops of BQC and let stand for 30 min.
9. Add 15 ml. neutral n-butanol, shake and centrifuge at 1450 rpm. for a few minutes.
10. Remove some of the alcoholic layer and read the color intensity in a Klett-Summerson photoelectric colorimeter with a 645 mμ wave-length filter.

Controls with 1 ml. distilled water added instead of the material to be tested were made simultaneously. The control readings were subtracted from the samples. The results are

recorded as micrograms of phenol liberated per milliliter of product per hour. A standard curve (Figure 5) was made, as suggested by Sanders and Sager, using pure phenol.

Determination of phosphatase activity of cheese at pH 5.4

The steps used for determining phosphatase activity in cheese at pH 5.4 were as follows:

1. Grind 1 gm. of freshly cut cheese in a mortar with AMP buffer without disodium phenylphosphate added and warmed to 40° C. Make quantitatively to 10 ml.
2. Add 1 ml. of the cheese-buffer mixture to 10 ml. of AMP buffer-substrate and proceed as for milk and whey but double the number of inversions of the tubes while in the water bath. The results are recorded as micrograms of phenol liberated per gram of cheese per hour.

Preparation of cheese for determination of nitrogen fractions

Knudsen and Overby (1942) showed that more uniform and reproducible values of nitrogen distribution in cheese could be obtained by first dissolving the cheese in a sodium citrate solution and then determining the different nitrogen fractions on this cheese solution, rather than determining the different fractions directly on the cheese.

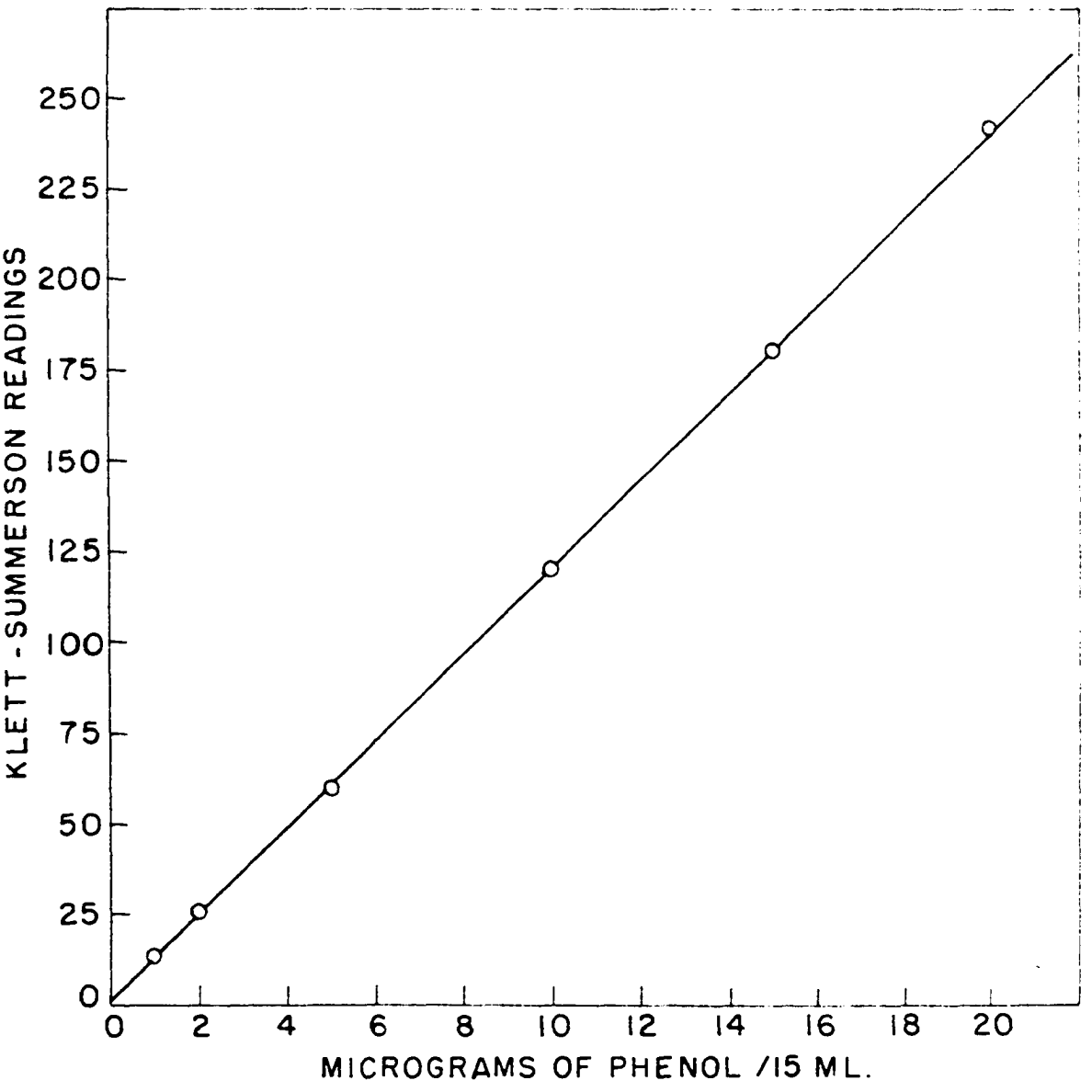

Figure 5. Standard curve for conversion of Klett-Summerson readings to micrograms of phenol per 15 ml.

Eight gm. of finely shredded cheese was weighed into a beaker and 50 ml. of 10 per cent sodium citrate solution added. The beaker was placed in a water bath at 40° C. When all the cheese had been dissolved, the solution was transferred quantitatively to a 200 ml. volumetric flask and made to volume with distilled water.

Total nitrogen was determined by the Kjeldahl method, using 20 ml. of the cheese solution.

Soluble nitrogen was determined after removal of the insoluble protein fraction. Fifty ml. of the cheese solution was transferred to a 200 ml. volumetric flask and enough 0.2 N acetic acid added to bring the pH down to 4.6 ±0.1. After making to volume with distilled water and mixing, the contents were filtered through Whatman no. 12, 18.5 cm. folded filter paper and nitrogen determined on 100 ml. of the filtrate by the Kjeldahl method.

Ammonia nitrogen was determined by pipetting 50 ml. of the cheese solution into a Kjeldahl flask. After addition of 250 ml. of water and 2 gm. of MgO the NH_3 was distilled off. The results are presented as total, soluble and ammonia nitrogen in per cent of the cheese and as soluble and ammonia nitrogen in per cent of total nitrogen.

Preparation of cheese extract for paper chromatography

The method of Kosikowsky (1951a) was used in principle to extract the free amino acids from the cheese, but as smaller chromatograms were used, it was found advantageous to make a more concentrated amino acid extract and apply smaller amounts than used by Kosikowsky.

Ten gm. of cheese were blended for 1 min. with 30 ml. of distilled water at 40° C. in a Waring blendor fitted with a monel metal jar of semi-micro size, and then made to 50 ml., using the remaining water to rinse out the jar. The solution was transferred to a 125 ml. erlenmeyer flask and heated to 75° C. for 5 min. with agitation, cooled to room temperature and filtered through Whatman no. 12 folded filter paper. Five ml. of the filtrate were mixed with 15 ml. of 95 per cent ethyl alcohol and left standing for a few minutes until a definite precipitate had formed and then filtered through Whatman no. 42 filter paper. Ten ml. of the filtrate were placed in a small beaker and evaporated to dryness over $CaCl_2$ in a vacuum desiccator.

The residue was dissolved as well as possible in 0.4 ml. distilled water, using a rubber policeman to dislodge the material from the side of the container. To prevent possible interference on the chromatogram, the undissolved substances were removed by centrifugation at 10,000 rpm. for 10 min.,

as suggested by Storgårds and Lindquist (1953a).

Paper chromatography procedure

The two-dimensional procedure employed by Levy and Chung (1953) using buffered papers was adapted to suit the equipment on hand. Levy and Chung recommended using Whatman no. 52 filter paper but gave an alternate procedure for Whatman no. 1 filter paper. No. 52 paper was not available until the time of the 6 months examination, so no. 1 paper was used to examine the fresh, 1 and 3 months old cheese.

The paper was cut to 8.5 X 10.5 inch size and 0.025 ml. of the amino acid solution representing 0.03 gm. of cheese applied with 0.01 and 0.005 ml. pipettes to a spot 1 inch from the bottom and 2 inches from the left hand side. The spot was placed over a microscope lamp for quick drying. As the ascending method of developing the amino acid spots was used, the paper was rolled up and stapled together without the edges touching. The containers used for development of the chromatograms were 4 qt. Dazy churns 9 inches high. The 4.5 inch opening was covered with aluminum foil held tight with rubber bands. The first solvent the paper was placed in was the top layer of a mixture of butanol:acetic acid:water (4:1:5). The bottom layer of this mixture was placed in a beaker in the bottom of the churn. A wick of filter paper, extending to the

top of the churn, was placed in the beaker to provide for better saturation of the atmosphere with the components of the watery layer. The solvent was allowed to reach the top, whereupon the paper was removed and dried under the hood with a hot plate in front for at least 2 hr.

After drying, the paper was placed between two strips of glass to cover the amino acid spots and sprayed with 0.1 M borate buffer (pH 8.3 for Whatman no. 1 paper and pH 9.3 for Whatman no. 52) and dried again for at least 0.5 hr. One inch of paper was cut off the sides, to remove irregularities caused by the staples, leaving an 8.5 X 8.5 inch sheet. The paper then was stapled in the opposite direction and placed in the second solvent system.

Phenol with 20 per cent buffer added was found more advantageous than phenol plus cresol as the second solvent system. Levy and Chung recommended a mixture of cresol and phenol for more even distribution of the amino acid spots, but showed also that more spreading could be obtained in the area between aspartic acid and alanine by using phenol alone. As serine, which is of particular interest in this study, is located in this area, it was decided to use phenol alone as the developing agent. The developing agent was contained in a 3.5 inch petri dish located in the center of the churn. The churn was lined with filter paper and a portion of a mixture of 250 ml. buffer and 8 ml. of the developing agent

placed in the bottom.

The solvent was allowed to reach the top and the chromatogram was removed and dried as before for at least 2 hr. The colored areas were developed by spraying the paper with a mixture of 50 ml. of 0.1 per cent ninhydrin in ethyl alcohol, 2 ml. of collidine and 15 ml. of glacial acetic acid and then holding the wet paper 2-3 inches above a hot plate in a hood.

Standard chromatograms were prepared as recommended by Levy and Chung.

The size and intensity of the spots are recorded by means of one to four plusses. The complete absence of an amino acid compared to other cheeses in the same examination period is indicated by a negative sign. Comparison of spots is most accurate within a lot but can be made with some degree of accuracy for the entire examination period and from period to period.

Determination of pH on cheese

Measurements of pH on cheese were made with a Leeds and Northrup quinhydrone electrode and saturated calomel half cell, using a mixture of quinhydrone and cheese into which the noble electrode was inserted.

RESULTS

Deamination of Amino Acids by L. casei

Preliminary studies and observations

Production of cells. Because of the inhibitory effect of carbohydrates on bacterial deaminases, a medium of a composition similar to that listed under Experimental Methods, but free of carbohydrate and without V-8 juice added, was first selected for production of cells. However, such a medium could not support satisfactory growth of L. casei. The pH of the medium would only decrease from 7.0 to approximately 6.0 after 16 hr. incubation at 32° C. and the yield of cells was very low. Prolonged incubation did not produce any additional decrease in pH. An attempt to overcome this deficiency was made by adding filtered V-8 juice, which on many occasions had been demonstrated to increase growth of lactobacilli. Addition of 10 per cent V-8 juice increased the yield of cells tremendously, and also gave a satisfactory rate of growth with a decrease in pH from 7.0 to approximately 4.2, using a 0.1 per cent inoculum and 16 hr. at 32° C.

Dried cells were considered for use in all the experiments requiring cells grown under standard conditions, in order to

eliminate quantitative differences in activity which occurred between small batches of cells. Such differences occurred in spite of apparently identical conditions of production of the individual small batches. Cells dried under vacuum over Drierite showed high activity when just removed from the desiccator, but lost their activity so quickly it was considered more feasible to use freshly grown cells for each experiment, a procedure which was adopted.

Selection of a suitable strain of L. casei. L. casei strains 25, 28, 142 and 7 were investigated for their ability to deaminate DL-serine. Deamination was carried out at pH's 8.3 and 5.4 under standard conditions for 1 hr. at 37° C. A pH of 8.3 was selected as being optimum for bacterial deaminases and pH 5.4 as within the range of pH of ripening cheese.

Table 1 shows L. casei strain 7 deaminated DL-serine at a higher rate than did the other strains. On the basis of this, strain 7 was selected for further studies. Another distinguishing characteristic of this strain was its ability to produce H_2S in the growth medium. This could easily be detected by smell or by lead acetate paper.

Table 1

Deamination of DL-serine by L. casei strains 25, 28, 142 and 7

Strain no.	Mcg. NH_3 released per mg. bacterial N at: pH 5.4	pH 8.3
25	2.0	3.2
28	-2.5	6.2
142	-3.0	4.8
7	11.2	20.3

(Incubated for 1 hr. at 37° C.)

Deamination temperature. Optimum temperature for growth of L. casei strain 7 was 37° C. and, until temperature studies on deamination of serine had been conducted, 37° C. also was considered to be the optimum temperature for deamination. When temperature studies showed optima at 46° C. for deamination at pH 8.3 and at 52° C. at pH 5.4 (Figure 8), the studies which already had been carried out at 37° C. were repeated at the higher temperatures. The results at 37° C. when available were included in the following experiments when they were considered helpful for the presentation. After the optimum temperatures had been established, all further studies were carried out at these temperatures.

Effect of time. The preliminary investigations included studies to determine the relationship between time of deamina-

tion *in vitro* and release of NH_3. Cells of *L. casei* strain 7 were allowed to deaminate DL-serine in a series of tubes. Tubes were removed at 1 hr. intervals and analyzed for NH_3.

The results, as shown in Figure 6, indicated similar trends at the different pH's and temperatures, with a comparatively straight line relationship up to 2 hr. and then a leveling off or decline of deaminase activity. The decrease in deaminase activity after 2 hr. could be due to either exhaustion of the substrate, loss of some vital substance or establishment of equilibrium. The tubes at pH 8.3, 46° C. and pH 5.4, 52° C. each contained 2.02 mg. bacterial nitrogen and the two tubes at 37° C., 1.82 milligrams each. The maximum actual amount of NH_3 liberated at pH 8.3, 46° C. was 210 mg. This was approximately 50 per cent of the NH_3 , which theoretically could be released from 0.5 ml. of 0.05 M DL-serine. The cessation of deamination could then indicate that D-serine is not deaminated by *L. casei*. However, the curve at pH 5.4, 52° C. and the two lower curves show the same effect of time while less than half of the theoretical amount of L-serine had been deaminated. This could indicate equilibrium had been reached or the loss of some vital substance had occurred. The decrease in NH_3 after 2 hr. at the higher temperatures could indicate that NH_3 is consumed by *L. casei*.

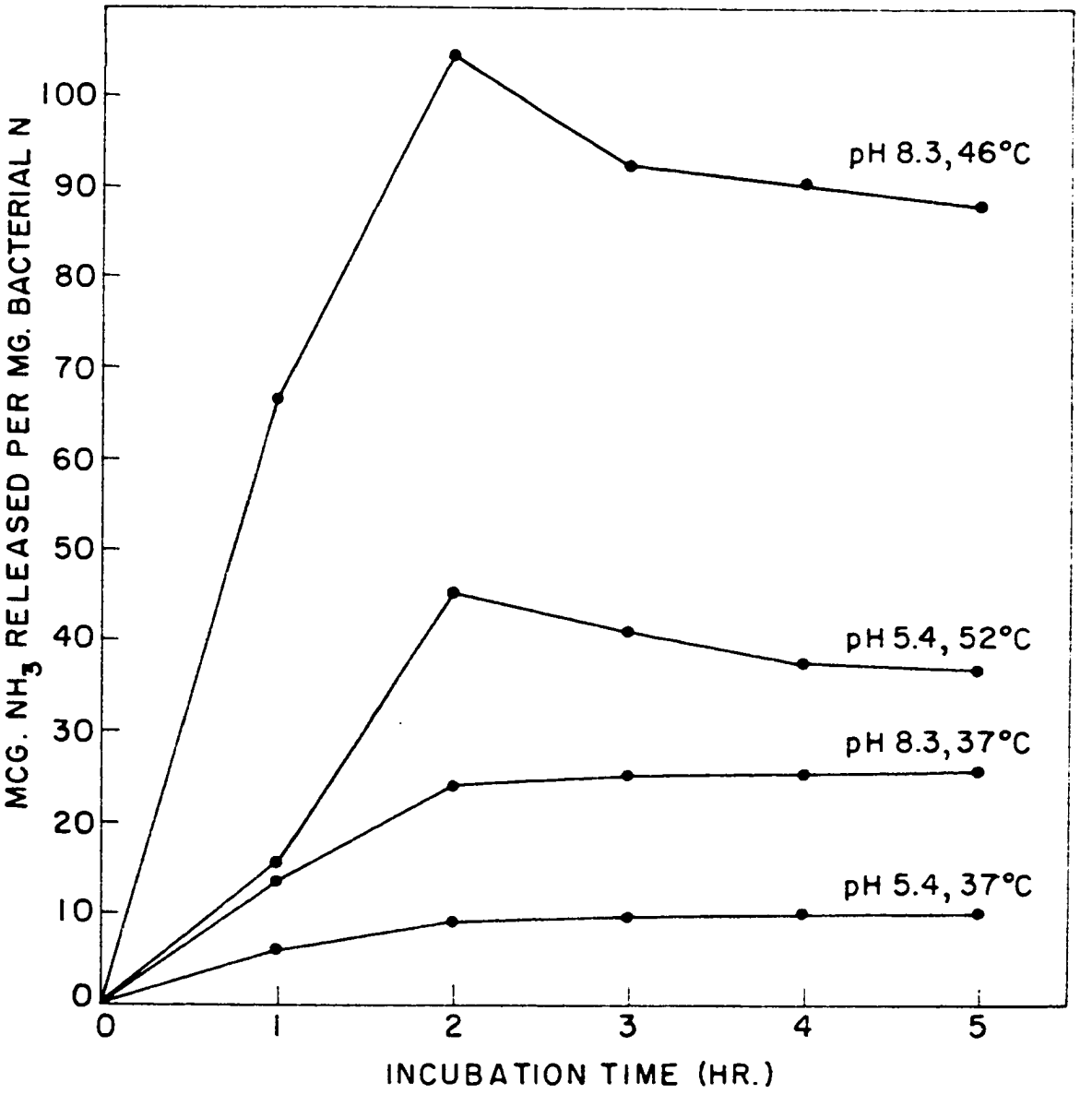

Figure 6. Effect of incubation time on deamination of DL-serine by L. casei strain 7 under aerobic conditions.

In view of the relatively straight-line relationship of time and NH_3 released up to 2 hr., it was decided to let deamination take place for 2 hr. in the different experiments.

Formation of pyruvic and lactic acids. Deamination of amino acids can be followed by measuring the increase in the resulting products, either the NH_3 or the keto acids, pyruvic acid in the case of serine. In this study, as already shown, deamination was followed by measuring the increase in NH_3, but increases in pyruvic acid and, what could be expected with L. casei, lactic acid also were demonstrated with serine. Table 2 demonstrates increases in NH_3 and pyruvic acid and NH_3 and lactic acid of two independent runs at 37° C.

Table 2

Formation of NH_3, pyruvic acid and lactic acid on deamination of DL-serine by L. casei strain 7

Run	Mcg. product formed per mg. bacterial N* at:					
	pH 5.4			pH 8.3		
	pyruvic acid	lactic acid	NH_3	pyruvic acid	lactic acid	NH_3
I	6.9	-	11.8	12.0	-	18.4
II	-	4.7	8.6	-	11.0	16.4

(Incubated for 1 hr. at 37° C.)

*Run I: 1.87 mg. bacterial N per tube.
*Run II: 1.59 mg. bacterial N per tube.

The results could indicate a relationship between increase in NH_3 on one hand and increases in pyruvic and lactic acids on the other. Unfortunately, data which would permit balancing of the fermentation equation were not obtained.

The choice of phosphate buffer. After some of the preliminary studies had been conducted, it became apparent some consideration must be given the buffer. Some aspects of the investigation were expected to cover a rather wide range of the pH scale. The desirability of being able to use the same buffer over the whole pH range to be investigated was quite obvious. Phosphate buffer has been widely used in physiological studies but has only little buffering capacity outside pH 5.8 to 8.0. Therefore, acetate, 2-amino-2-methyl-1,3-propanediol, with a buffer capacity ranging from pH 4.0-9.5, was considered.

Tests were made to compare the two buffers at pH's ranging from about 4.5-10.0. About equal amounts of NH_3 were released from DL-serine in the acid range, but the AMP buffer showed a marked inhibitory effect in the alkaline range, compared to the phosphate buffer. This inhibitory effect could not be overcome by addition of phosphate buffer to the AMP buffer.

Measurements of pH of the phosphate buffer-substrate-cell mixture showed a decrease in pH in the alkaline range after 2

hr. incubation. The decrease ranged from about 0.7 units at pH 9.8 to almost none at pH 7.4. At pH 8.3 the decrease was only a few tenths of a unit. In the acid range the pH of the mixture increased about 0.6 units at pH 4.5 and none at pH 5.8. The increase at pH 5.4 was only one or two tenths of a unit.

Serine, which was to be used as substrate in most of the studies, has a pK_1 of 2.21 and a pK_2 of 9.15. This shows that serine can act as a buffer from pH 1.2 to 3.2 and from pH 8.2 to 10.2 and thus should lend some buffering capacity to the deamination systems in the alkaline range.

The phosphate buffer was regarded as a better choice than the AMP buffer because of the inhibitory effect of the latter in the alkaline range. The disadvantage of phosphate buffer in not being able to maintain pH over the entire range investigated was considered smaller than the disadvantage of the inhibitory effect of the AMP buffer in the alkaline range.

The pH's at which deaminations have been recorded to take place in this report are the pH's of the buffer-substrate-cell mixtures before incubation.

Phosphoserine composition. Phosphoserine was isolated from casein as the barium salt by the method mentioned under Experimental Methods and analyzed for barium, total phosphorus and total nitrogen. The analytical results are listed below,

together with the calculated theoretical results.

Calculated:	9.69% P,	4.38% N,	42.9% Ba
Found:	9.59% P,	4.36% N,	38.6% Ba

Deamination of individual amino acids and some related compounds

Most of the common amino acids plus phosphoserine, glutamine and asparagine were subjected to deamination by L. casei strain 7. Amino acids not used were hydroxyproline, which is not present in casein (Gordon et al., 1949), and tyrosine and cystine, which are essentially insoluble in water.

Some of the amino acids used were the L-isomers and some mixtures of D- and L-isomers, in the form in which they commonly are commercially available. Phosphoserine was prepared as mentioned under Experimental Methods. As was the case with the substrate solutions of serine, the individual amino acid solutions were made 0.05 M with regard to the amino acid and adjusted to pH of the buffer before being made to volume. In each case 0.5 ml. of solution was used as substrate. The results as listed in Table 3 are representative and were reproducible. Some variation existed between different runs as to the absolute values of NH_3 released, but

the values of NH_3 released from amino acids showing little release of NH_3 were always low and the values of NH_3 released from cysteine and serine and asparagine in particular always were high.

Most of the amino acids were not deaminated or were deaminated only to a very low degree (less than 10 mcg. of NH_3 per mg. of bacterial N), at either pH 5.4 or pH 8.3 at the different temperatures. L-cysteine was deaminated to some extent, with the most NH_3 released at pH 5.4, 52° C. L-serine was deaminated at approximately twice the rate of DL-serine, particularly at the optimum temperatures. Phosphoserine was not deaminated. Threonine, the other hydroxyamino acid, was deaminated slightly.

Ammonia also was released from L-asparagine at approximately the same rate as from L-serine at pH 8.3, 46° C. but at a lower rate at pH 5.4, 52° C. and at 37° C. for both pH's. The NH_3 probably was released from the amide group of asparagine because aspartic acid was not deaminated. Release of NH_3 from an amide group, like deamination of serine and cysteine, is a hydrolytic reaction and no oxygen is involved.

Glutamine is not included in Table 3. The substrate controls showed NH_3 was released spontaneously from glutamine at a very high level. This cast doubt upon the validity of the net result. None of the other amino acid solutions showed appreciable spontaneous release of NH_3.

Table 3

Deamination of individual amino acids and some related compounds by L. casei strain 7

Substrate	Mcg. NH_3 released per mg. bacterial N* at:			
	37° C.		52° C.	46° C.
	pH 5.4	pH 8.3	pH 5.4	pH 8.3
L(+)-lysine mono HCl	0.6	4.6	2.2	0.1
L(+)-histidine·HCl	3.8	-0.1	0.6	-2.3
Glycine	0.1	0.1	0.6	0.0
DL-valine	-3.1	5.0	6.8	-1.1
DL-methionine	-0.4	-0.1	7.8	-3.1
L-tryptophan	0.0	0.2	6.6	0.4
L-leucine	-3.5	4.7	7.1	-2.2
DL-phenylalanine	2.8	1.2	5.4	-2.4
L(-)-proline	4.1	-3.7	4.3	-2.0
L(+)-glutamic acid	-3.4	-2.6	-4.0	3.0
DL-aspartic acid	-2.6	1.4	-5.3	2.7
L-asparagine	0.0	4.8	15.5	212.6
DL-threonine	0.0	5.7	5.3	10.0
L-cysteine·HCl	8.4	7.3	23.2	12.6
L-arginine·HCl	3.8	0.8	-4.0	1.0
DL-isoleucine	0.0	1.7	-6.7	4.5
DL-alanine	-1.2	0.0	-1.2	0.7
DL-serine	9.3	19.2	29.9	108.4
L-serine	13.1	29.3	65.2	231.8
Phosphoserine	0.0	0.0	0.0	-1.0

*37° C.:1.99 mg., 52 and 46° C. 1.56 mg. bacterial N per tube.

The subsequent experiments were carried out to study some of the factors influencing deamination of serine by L. casei.

Factors influencing production of the serine deaminase of L. casei strain 7

Effect of glucose in the growth medium on deamination of DL-serine and formation of H_2S. As mentioned previously, addition of carbohydrate to the growth medium has been found to be inhibitory to formation of deaminases by other bacteria. To check on a similar inhibitory effect on L. casei strain 7, this bacterium was grown in the presence of various amounts of glucose.

V-8 juice contains a certain amount of carbohydrates. The usual addition of 10 per cent V-8 juice to the medium was estimated to give a carbohydrate concentration of approximately 0.2 per cent. In order to have more defined conditions with regard to carbohydrate concentration, strain 7 was grown in a series of media of the usual composition, but without V-8 juice added. Instead 0.2-2.0 per cent glucose was added. For comparison a culture was also grown in the regular V-8 juice medium.

After 16 hr. at 32° C. the cultures were checked for pH and H_2S production and the cells were harvested and checked

for deaminase activity. H_2S was checked by smell and by lead acetate paper and recorded as to relative concentration. Representative results are listed in Table 4.

Table 4

Effect of the concentration of glucose in the growth medium on formation of H_2S and deamination of DL-serine by L. casei strain 7

Addition to medium	Final pH of culture	H_2S	Mg. bact. N per tube	Mcg. NH_3 released per mg. bacterial N at: pH 5.4, 52° C.	pH 8.3, 46° C.
0.2% glucose	4.57	+	0.84	15.3	47.7
0.5% "	4.27	+++	0.95	24.0	108.8
1.0% "	4.19	++	1.13	18.4	77.8
1.5% "	4.15	+(+)	1.35	16.0	75.0
2.0% "	4.21	-	1.28	9.9	52.9
10% V-8 juice	4.19	+++	1.34	48.4	171.3

The media containing glucose did not support growth of L. casei strain 7 as well as the medium containing V-8 juice. In general the yield of cells increased with the concentration of glucose, but the V-8 juice medium yielded approximately twice as many cells as did the best glucose-containing medium, as judged by wet weight. The cells from the V-8 juice medium were diluted more than the others to get a cell concentration in a similar range.

Addition of 0.2 per cent glucose was not quite enough to support full growth of strain 7, as judged by the final pH of the medium. Addition of 0.5-2.0 per cent glucose resulted in final pH's comparable to that of the V-8 juice medium. The lower concentration of H_2S in the medium with 0.2 per cent glucose added probably is due to the higher pH. Otherwise the concentration of H_2S formed was inversely proportional to the glucose concentration. With 0.5 per cent glucose added, about the same amount of H_2S was formed as in the V-8 juice medium. As the sugar concentration increased, the concentration of H_2S decreased and none was formed in the medium with 2 per cent glucose added.

The ability of the cells to deaminate DL-serine also was affected by glucose in the growth medium. As the concentration of glucose increased, less NH_3 was released at both pH 5.4 and 8.3, but even with 2 per cent glucose added to the medium, the cells showed deaminase activity.

Apparently the ability of the cells to deaminate serine also depends in part on the physiological state or the age of the cells. The cells grown in the medium containing 0.2 per cent glucose were harvested at pH 4.57 and showed less deaminase activity than cells grown in the medium containing 0.5 per cent glucose and harvested at pH 4.27, although the results with from 0.5 to 2.0 per cent glucose in the medium indicated depression of enzyme formation with increasing

glucose concentration. The effect of the physiological state of the cells on their ability to deaminate serine is covered in a subsequent experiment and the effect of the age of the cells is covered in a later experiment on stability of the enzyme.

Comparison of the results for release of H_2S in the growth medium and the release of NH_3 by deamination of DL-serine at the different glucose concentrations indicates a possible connection between the two reactions. The largest decreases in release of H_2S at the different levels of glucose corresponded to the largest decreases in deaminase activity. Another evidence of a relationship between the two reactions was obtained when L. casei strain 7 for a short period for some unknown reason failed to produce H_2S in the growth medium and also lost the ability to deaminate DL-serine.

V-8 juice apparently contains some factor(s) besides carbohydrates, which supports growth of L. casei. The cells grown in the V-8 juice also exhibited much more deaminase activity than any of the others. Whether this was a direct effect of the V-8 juice or an indirect effect because a lower pH was obtained with a lower sugar concentration, would be difficult to say; it is not known how much NH_3 the cells grown with 0.2 per cent glucose in the medium would have been able to release had they been able to lower pH of the medium to a level similar to that in the V-8 juice medium. Experiments

on lowering of pH by addition of lactic acid were not carried out.

Deaminase activity of cells harvested at different physiological states as expressed by pH of the medium at harvest time.

The experiments on effect of glucose and effect of pasteurization indicated that the physiological state at which the cells were harvested affected their ability to deaminate serine. These experiments also were used to demonstrate the stimulatory effect of anaerobic-reduced conditions. (The experiments on effect of pasteurization and effect of anaerobic-reduced conditions are covered in the following section on factors influencing deamination of DL-serine by L. casei strain 7.)

To further investigate this aspect, six flasks of medium were inoculated at intervals of about one hour with L. casei strain 7 and incubated at 32° C. When the oldest culture was 16 hr. old, all the cultures were checked for pH and the cells were harvested and checked for deaminase activity against DL-serine under aerobic and anaerobic-reduced conditions. The pH's of the cultures, which were taken as indexes of different physiological states, ranged from 5.61 to 4.23, the latter pH being about normal for 16 hr. of incubation. The results in

Figure 7 show that the ability of the cells to deaminate DL-serine under aerobic conditions increased at both pH 5.4 and 8.3 as the cells grew older physiologically (decreasing pH of the medium at harvest time). Tested under anaerobic-reduced conditions, the deaminase activity of the cells decreased as the cells grew older physiologically, particularly when tested at pH 8.3. The amounts of NH_3 released were always higher under anaerobic-reduced test conditions at similar states. The difference was particularly large for younger cells which showed a high amount of activity under anaerobic-reduced test conditions and only little under aerobic test conditions.

The results at both pH 5.4 and 8.3 with cells harvested at pH 4.23 and 4.32 were in good agreement with the results found when effect of pasteurization was studied.

The large amount of NH_3 released per milligram of bacterial nitrogen at pH 8.3 under anaerobic-reduced conditions with cells harvested at pH 5.61 and 5.18 was obtained with comparatively little bacterial nitrogen per tube. Because it was considered important to avoid any difference in treatment and holding time of the different cultures during harvesting and washing, the same amount of medium was inoculated for each culture, although the same yield of cells at the different pH levels could not be expected. The milligrams of bacterial nitrogen per tube ranged from 1.2 at pH 4.23 to 0.4 at pH 5.61.

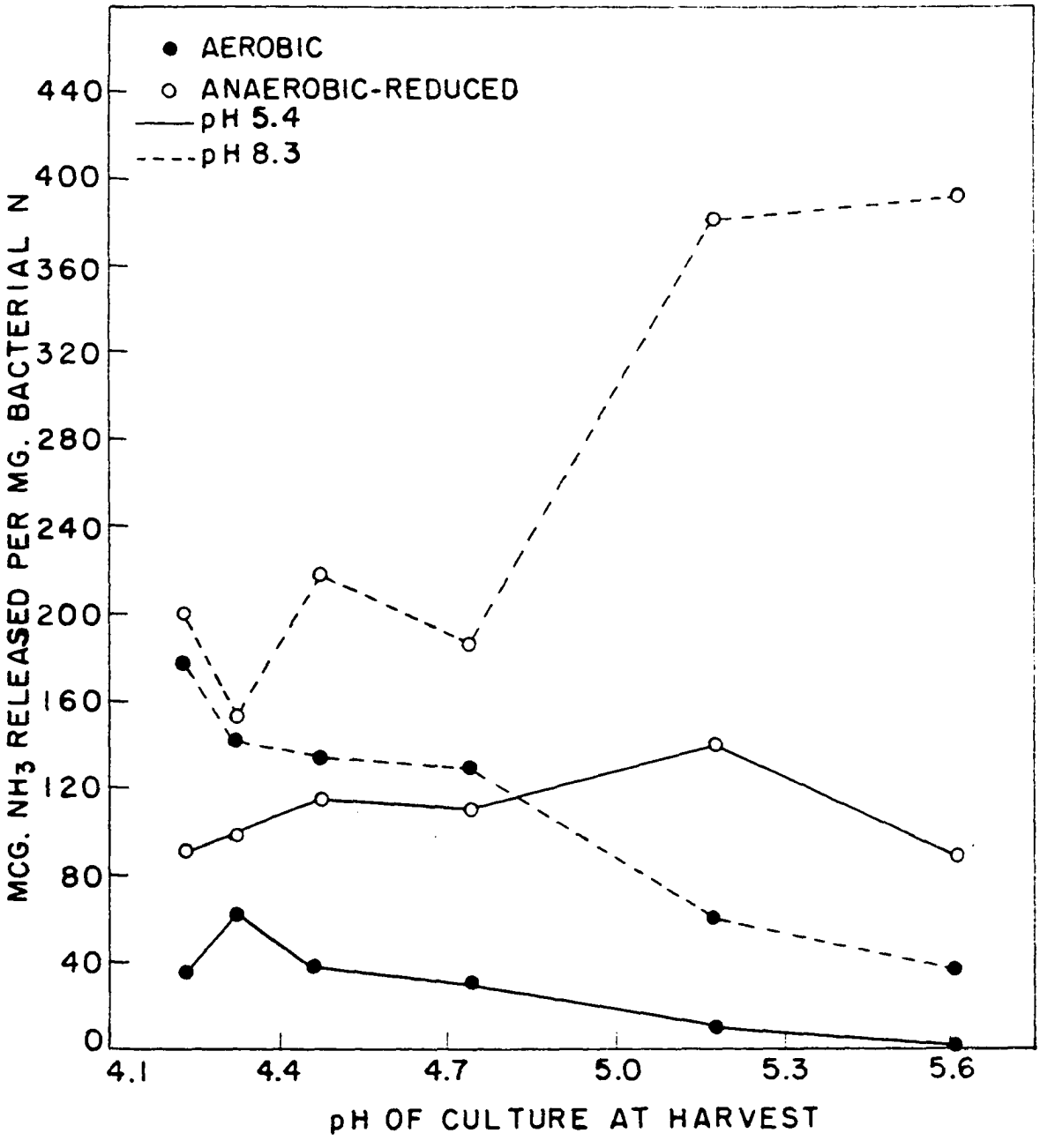

Figure 7. Effect of the physiological state of cells of L. casei strain 7, as expressed by pH of the culture at harvest, on deamination of DL-serine under aerobic and anaerobic-reduced conditions.

The actual amount of NH_3 released by the cells harvested at pH 4.23 was higher than that released by cells harvested at pH 5.61 but, on the basis of micrograms of NH_3 per milligram of bacterial nitrogen the results were reversed. An error may have been introduced regarding the relative level of deaminase activity of the cells, when harvested at different pH levels, due to the rather large difference in the amount of cells per tube. However, the results of deamination at pH 8.3 under aerobic and at pH 5.4 under both anaerobic-reduced and aerobic conditions did not indicate an increase in NH_3 released per milligram of bacterial nitrogen with the lower cell content per tube. The difference in the stimulatory effect of anaerobic-reduced conditions over aerobic at the different physiological states was unquestionable, as the tubes representing a given pH level all contained the same amount of cells. This difference was confirmed in the experiment on stability, which is to follow.

Factors influencing deamination of DL-serine by L. casei strain 7 grown under standard conditions

Effect of temperature. Tubes containing the buffer-substrate mixture were adjusted to the desired temperatures ranging from 5.5 to 60° C. before addition of the cells.

Figure 8 illustrates a typical example of the effect of temperature. Each tube contained 1.56 mg. of bacterial nitrogen and was incubated under aerobic conditions. The rate of deamination at pH 8.3 increased between 5.5 and 32° C. but the amounts of NH_3 released were comparatively low. From 32° C. the rate increased very rapidly with an optimum at 46° C. From 46 to 60° C. the rate of deamination decreased at about the same rate as it increased from 32 to 46° C.

Deamination at pH 5.4 showed the same general picture as at pH 8.3, except the rate was slower and the rapid increase in release of NH_3 did not take place until 46° C. and the optimum was reached at 52° C. rather than at 46° C.

Effect of pH. Determination of optimum pH for deamination of amino acids by other species of bacteria have always been carried out at the temperature considered optimum for growth of the species used. However, the previous results from temperature studies showed 37° C., optimum temperature for growth of L. casei strain 7, to be below optimum for deamination of DL-serine. The results also indicated the possible presence of two different enzyme systems with optima at 46 and 52° C. In view of this, optimum pH for deamination of DL-serine by L. casei strain 7 was determined at 37, 46 and 52° C. Typical results are illustrated in Figure 9. The tubes at 37° C. contained 1.91 mg. and the tubes at 46 and 52° C.

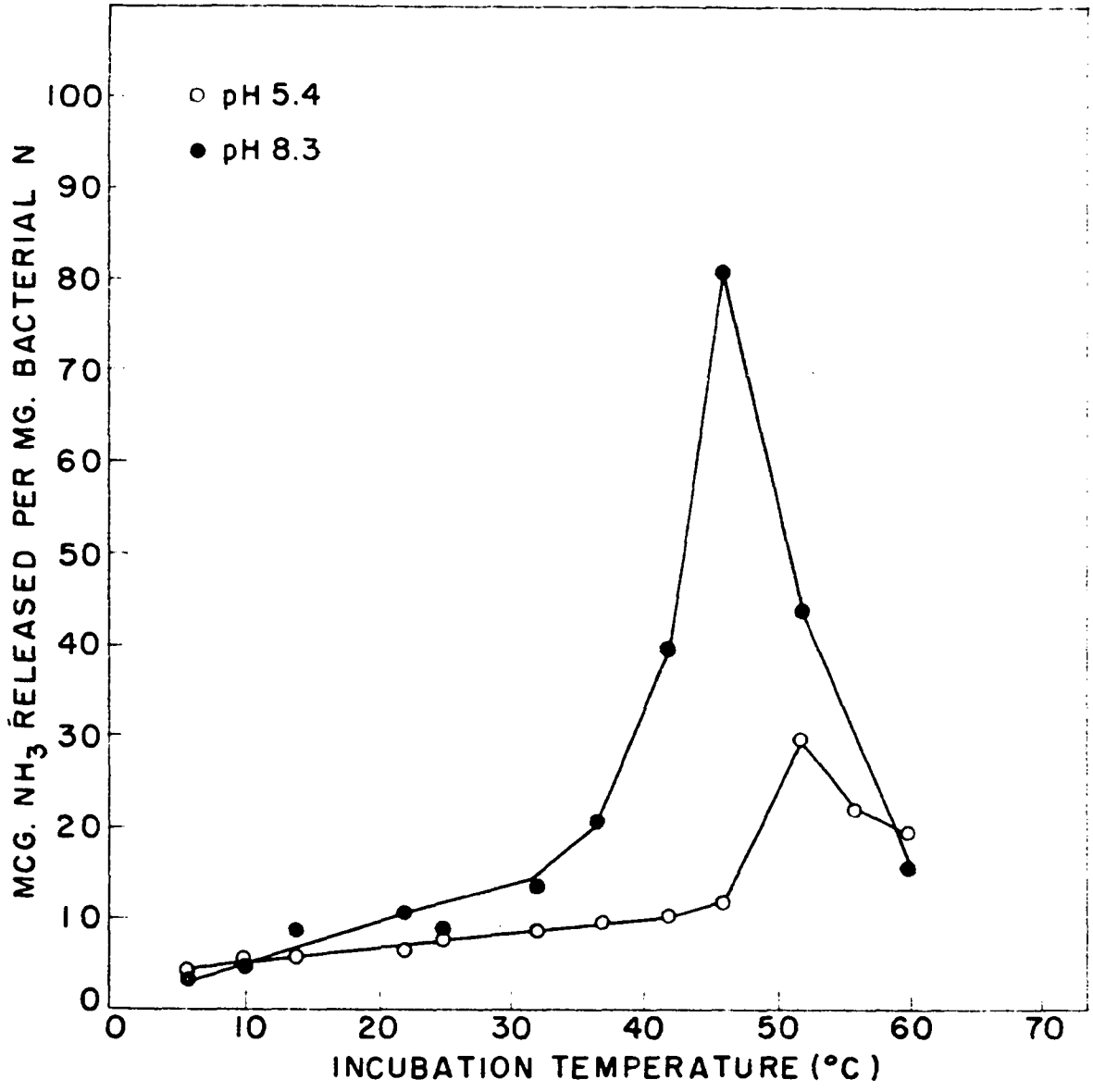

Figure 8. Effect of incubation temperature on deamination of DL-serine by L. casei strain 7 under aerobic conditions.

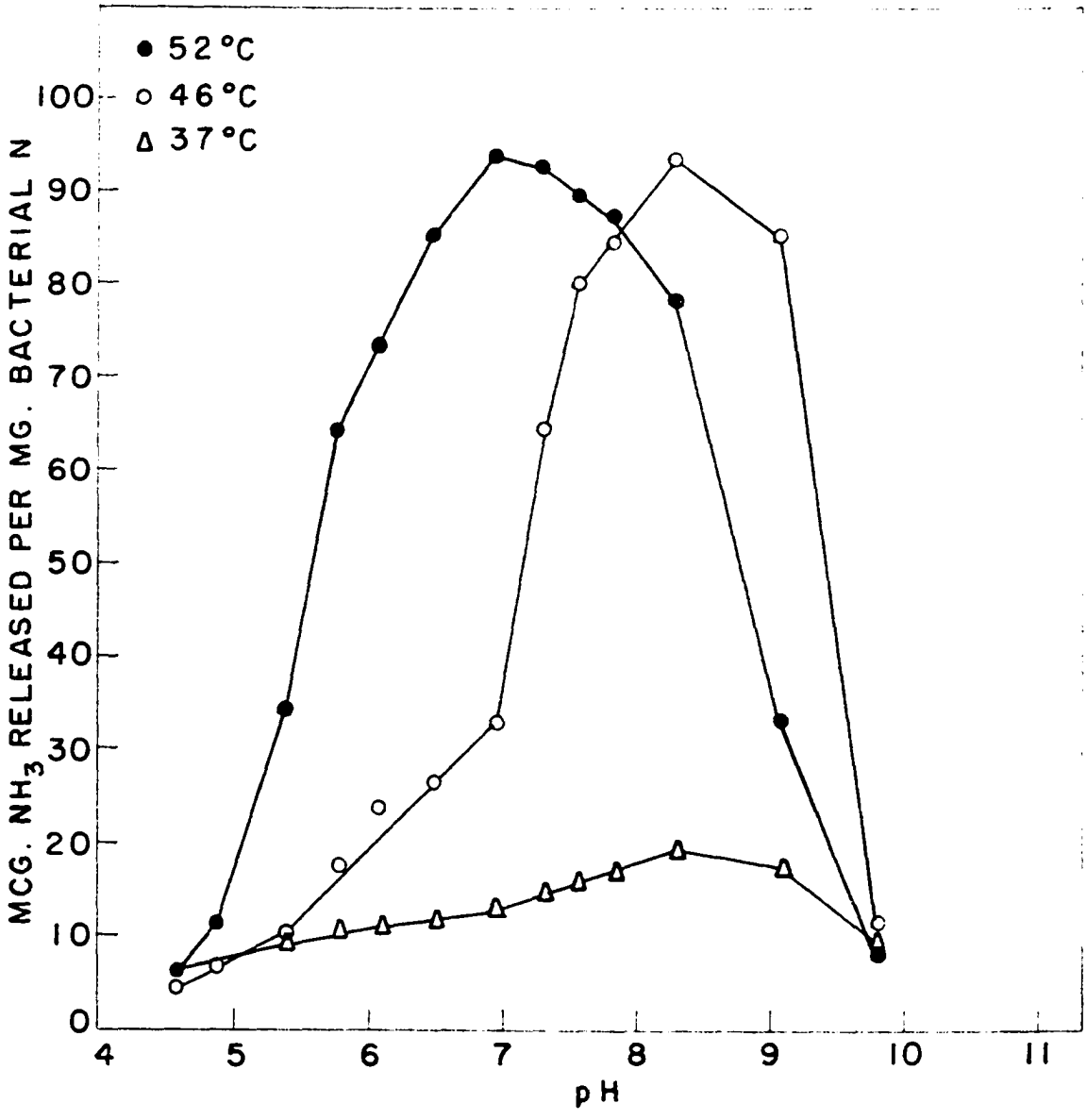

Figure 9. Effect of pH on deamination of DL-serine by L. casei strain 7 under aerobic conditions.

1.84 mg. of bacterial nitrogen per tube.

As was expected, different pH optima were obtained at 46 and 52° C. At 46° C. the optimum pH for deamination of DL-serine was about 8.3 and at 52° C. about 7, giving further evidence of the possibility of two enzyme systems, the two systems being about equally active against DL-serine at their optimum pH levels. The curve at 37° C. showed an optimum at pH 8.3 like the curve at 46° C., but a much lower level of activity was obtained.

It is interesting to note the break exhibited by all three curves around pH 7. The curve at 52° C. has the optimum at this pH and the curves at 46 and 37° C. both show a marked increase in activity as they pass into the alkaline range.

Effect of gassing with nitrogen and addition of cysteine, glutathione and adenosine-5-phosphate. The micro-environment in Cheddar cheese under which lactobacilli normally metabolize is reduced and anaerobic. It was thought such conditions might stimulate the serine deaminase activity of L. casei. Anaerobic conditions were established by gassing the tubes with nitrogen for 1 min. and reduced conditions by addition of 100 mcg. of L-cysteine as L-cysteine.HCl dissolved in 0.1 ml. water and adjusted to the pH of the buffer before use. Controls were treated in a similar manner. At the same time

reduced glutathione (GSH) and adenosine-5-phosphate (AMP) were tested for possible stimulating effect. Fifty mcg. of each were added per tube. Representative results are listed in Table 5. Gassing with nitrogen and addition of cysteine both resulted in an increase in NH_3 production, nitrogen gas having the most effect. Combined anaerobic and reduced conditions resulted in a still larger increase at pH 5.4, but did not effect the result at pH 8.3 over that of anaerobic conditions alone.

Addition of glutathione and adenosine-5-phosphate either singly or together seemed to have some inhibitory effect at pH 5.4, but the effect of glutathione at pH 8.3 was overcome in combination with adenosine-5-phosphate. Combination of either of these two compounds with nitrogen gas seemed to overcome their inhibitory effect. Values of NH_3 released, comparable to those of nitrogen gas alone, were obtained.

Deamination was allowed to go on for 4 hr. in separate tubes, under the conditions which had been shown to be most favorable, to find out if additional deamination would take place under such conditions beyond the 2 hr. period. The results in the last part of Table 5 indicate that no further deamination takes place even under anaerobic-reduced conditions.

The different responses at pH 5.4 and 8.3 to the different additions also support the hypothesis of two different enzyme systems.

Table 5

Effect of gassing with nitrogen and addition of cysteine, glutathione and adenosine-5-phosphate on the deamination of DL-serine by L. casei strain 7

Incubation time	Addition	Mcg. NH_3 released per mg. of bacterial N* at: pH 5.4, 56° C.	pH 8.3, 46° C.
	None	37.0	86.6
	100 mcg. cysteine	40.0	90.2
	Nitrogen gas	49.0	96.6
	Nitrogen + cysteine	56.4	95.8
2 hr.	50 mcg. GSH**	29.9	80.5
	50 mcg. AMP***	31.1	86.3
	GSH + AMP	28.6	86.8
	Nitrogen + GSH	46.6	96.2
	Nitrogen + AMP	50.5	99.6
	None	39.2	87.8
4 hr.	Nitrogen + cysteine	51.6	97.4
	Nitrogen + GSH + AMP	40.8	97.6

*1.83 mg. bacterial N per tube.
**GSH = glutathione.
***AMP = adenosine-5-phosphate.

Effect of pasteurization. Some strains of L. casei have been reported to survive heat treatment equal to pasteurization (Briggs, 1953 and Edmondson and Jensen, 1954). For this reason the effect of pasteurization on the ability of L. casei strain 7 to deaminate serine was studied.

Two cultures of L. casei strain 7 were grown under identical conditions in V-8 juice medium. After 16 hr. at 32° C. one of the cultures was neutralized to pH 6.7, heated to 61.8° C. and held at 61.8° C. ±1° C. for 30 min. and cooled. The other culture was cooled immediately to prevent any changes. Cells from both cultures were then harvested and washed as usual.

DL-serine was subjected to deamination by the pasteurized and unpasteurized cells under aerobic, anaerobic and anaerobic-reduced conditions. The two latter conditions were brought about by gassing with nitrogen and addition of 100 mcg. of cysteine, as in the previous experiment. The results in this particular study were calculated as micrograms of NH_3 released per milligram of original bacterial nitrogen, based upon the bacterial nitrogen content in the tubes containing the unpasteurized cells. Both types of cells underwent the same harvesting and washing procedure and were suspended in the same amount of water, but determination of bacterial nitrogen showed some of the proteinaceous material no longer was precipitated by centrifugation after neutralization and/or

pasteurization. The results are recorded in Tables 6 and 7.

Table 6

Effect of pasteurization on deamination of DL-serine by L. casei strain 7. Reaction at harvest time pH 4.20

Addition	Mcg. NH_3 released per mg. original bacterial N* at:			
	pH 5.4, 52° C.		pH 8.3, 46° C.	
	Unpast.	Past.	Unpast.	Past.
None	37.1	17.9	183.9	30.4
Nitrogen gas	72.2	69.1	199.9	179.9
Nitrogen + cysteine	81.3	102.5	200.0	179.0

*1.13 mg. bacterial N per tube.

Table 7

Effect of pasteurization on deamination of DL-serine by L. casei strain 7. Reaction at harvest time pH 4.35

Addition	Mcg. NH_3 released per mg. original bacterial N* at:			
	pH 5.4, 52° C.		pH 8.3, 46° C.	
	Unpast.	Past.	Unpast.	Past.
None	72.2	10.2	128.0	15.1
Nitrogen gas	96.7	36.9	141.3	73.3
Nitrogen + cysteine	109.3	49.7	142.8	110.0

*1.26 mg. bacterial N per tube.

The figures in Table 6 for the unpasteurized cells, confirm the results obtained in the preceding experiment. Deamina-

tion of DL-serine by L. casei strain 7 was stimulated by anaerobic conditions at both pH 5.4 and 8.3 and still further by anaerobic-reduced conditions at pH 5.4 but not at pH 8.3. Pasteurization reduced the deaminase activity of the cells about 50 per cent, when tested at pH 5.4 and about 80 per cent when tested at pH 8.3 under aerobic conditions. Under anaerobic-reduced conditions the pasteurized cells more than recovered their ability to deaminate DL-serine at pH 5.4.

Two separate experiments were included because the results in a repeat experiment (Table 7) indicated the physiological state at which the cells were harvested, as expressed by pH of the medium, influenced the deaminase activity of L. casei strain 7. The cells of Table 6 were harvested at pH 4.20, those of Table 7 at pH 4.35. Each test tube of the two runs contained approximately the same amount of bacterial nitrogen -- 1.13 mg. in Table 6 and 1.26 mg. in Table 7. The per cent reduction in bacterial nitrogen due to pasteurization was approximately 38 per cent in each case.

In general the results in Table 7 show the same trend as the results in Table 6. However, the unpasteurized cells in Table 7 showed more activity in the acid range and less in the alkaline under aerobic conditions, compared to Table 6. On the other hand, anaerobic-reduced conditions did not have as much stimulatory effect at pH 5.4 percentagewise on the cells harvested at pH 4.35. This was confirmed in the experiment on

effect of pH at harvest time.

Pasteurization affected the cells harvested at pH 4.35 much more than those harvested at pH 4.20, and deaminase activity, although greatly stimulated, was not fully recovered under anaerobic-reduced conditions. Pasteurization may actually have partially activated the enzyme activity of the cells harvested at pH 4.20, as indicated by results obtained at pH 5.4 under anaerobic-reduced conditions.

Stability. Some of the previous experiments showed that the physiological state and/or the age of the cells at harvest time influenced the deaminase activity of the cells. The following experiment was planned to give some further information about the affect of age on the stability of the enzyme.

Fifteen liters of V-8 juice medium were inoculated with L. casei strain 7 and incubated at 32° C. until the pH had decreased to 5.0. The culture was then cooled, mixed and divided into 500 ml. subcultures. One ml. of toluene was added to each subculture as a preservative. Toluene had previously been shown not to influence deamination.

The subcultures were divided into four groups. One group was adjusted to pH 4.2 with dilute HCl, pH 4.2 being the normal endpoint for growth in V-8 juice medium. The second group was left unadjusted at pH 5.0, a pH in the range of ripening Cheddar cheese. The third and fourth groups were

adjusted with dilute NaOH to pH's 7.0 and 8.3, these being optima for enzyme action on serine at 52 and 46° C., respectively.

One half of the subcultures at each pH level was closed with sterile cotton for the aerobic storage condition. The other half was stored under anaerobic-reduced conditions by adding 0.1 g. of cysteine·HCl to each flask and gassing for a few minutes with nitrogen, whereupon the flasks were quickly closed with sterile rubber stoppers and sealed with paraffin.

The subcultures were stored at 50° F., examined for pH after 24, 48 and 96 hr., and the cells harvested and checked for deaminase activity against DL-serine at pH 5.4 and 8.3 under aerobic and anaerobic-reduced conditions. The yield of cells was approximately 1.0 mg. of bacterial nitrogen per deamination tube. The subcultures also were checked for deaminase activity at 0 hr., right after having been adjusted to the different pH levels. These results were considered initial values for both aerobic and anaerobic-reduced storage conditions.

The changes in deaminase activity are illustrated in Figures 10, 11, 12 and 13. Figures 10 and 11 contain the changes which occurred in cells stored under aerobic conditions at the different pH levels when tested at pH 5.4, 52° C. and pH 8.3, 46° C., respectively. Figures 12 and 13 contain the changes which occurred under anaerobic-reduced storage

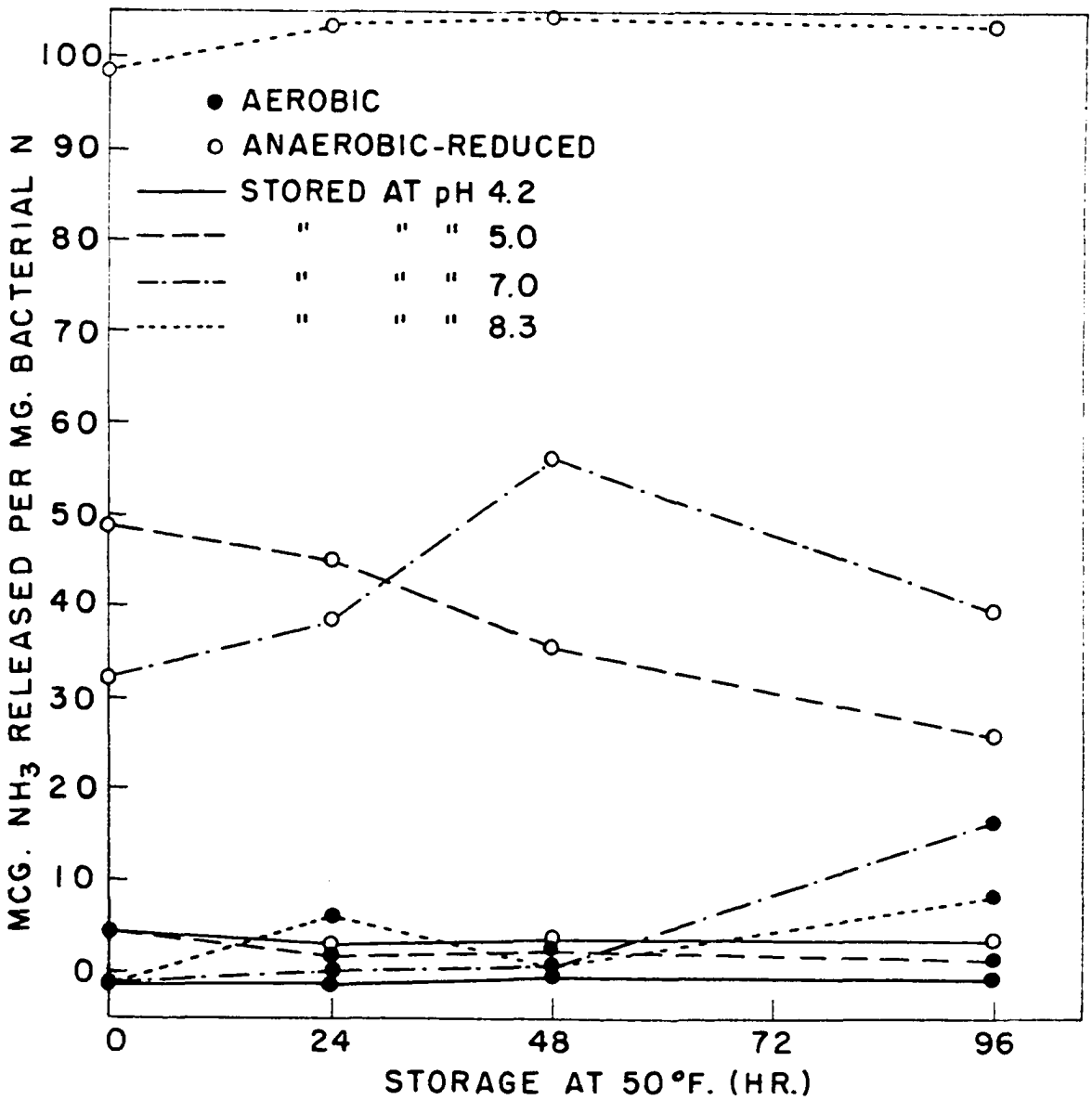

Figure 10. Effect of aerobic storage conditions at different pH levels of cultures of L. casei strain 7 on the ability of the cells to deaminate DL-serine. Deamination at pH 5.4, 52° C. under aerobic and anaerobic-reduced conditions

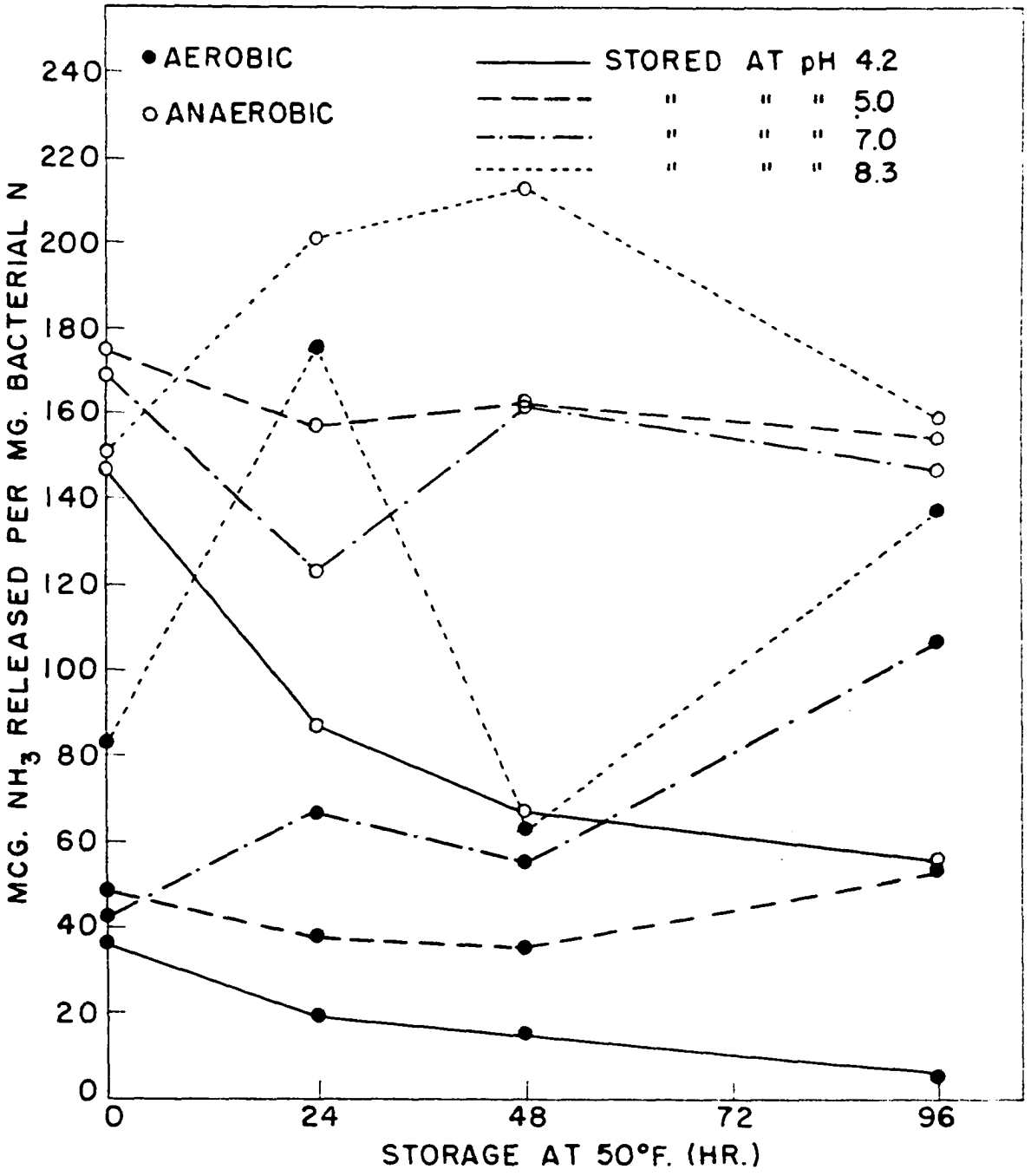

Figure 11. Effect of aerobic storage conditions at different pH levels of cultures of L. casei strain 7 on the ability of the cells to deaminate DL-serine. Deamination at pH 8.3, 46° C. under aerobic and anaerobic-reduced conditions

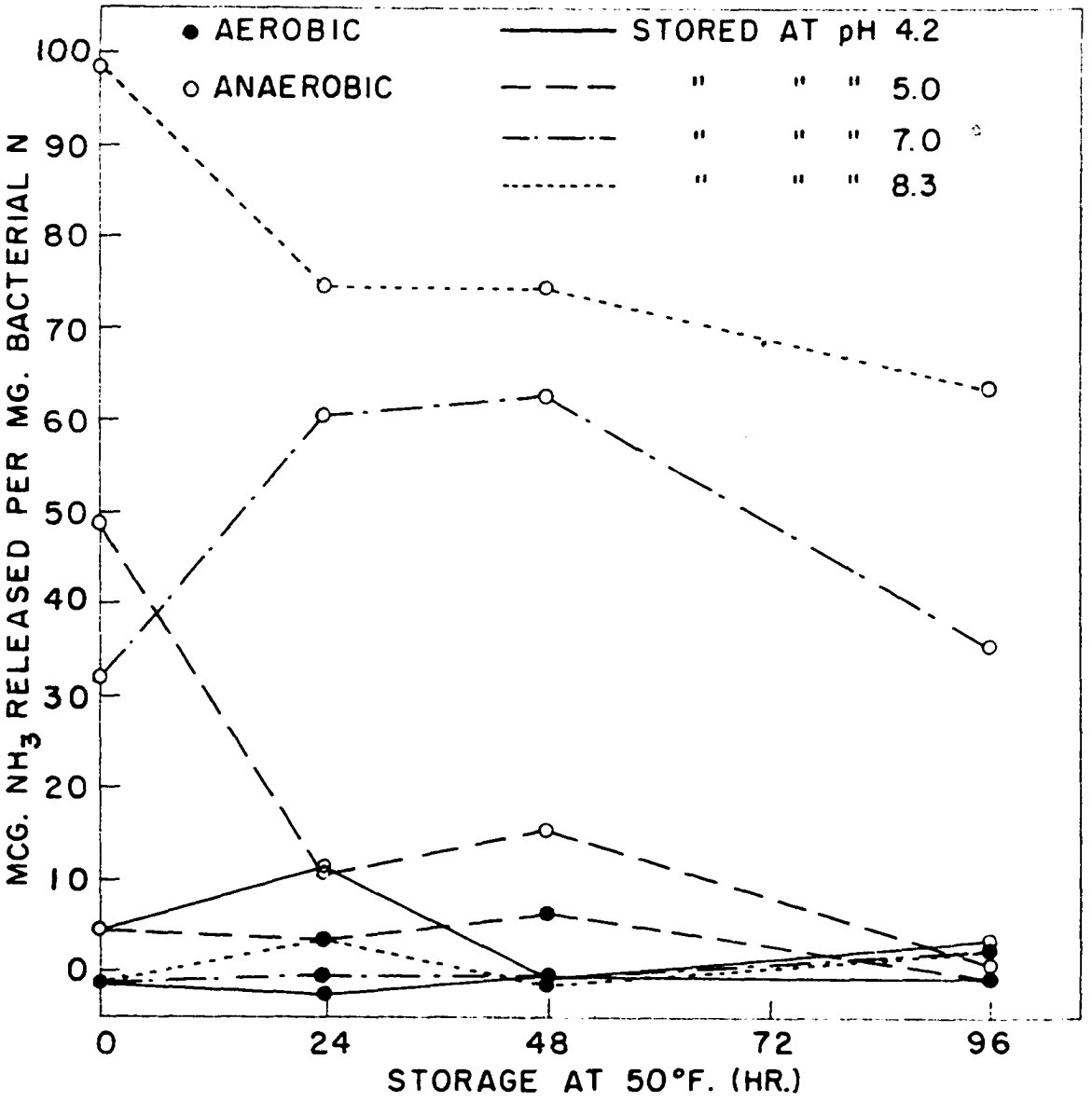

Figure 12. Effect of anaerobic-reduced storage conditions at different pH levels of cultures of L. casei strain 7 on the ability of the cells to deaminate DL-serine. Deamination at pH 5.4, 52° C. under aerobic and anaerobic-reduced conditions

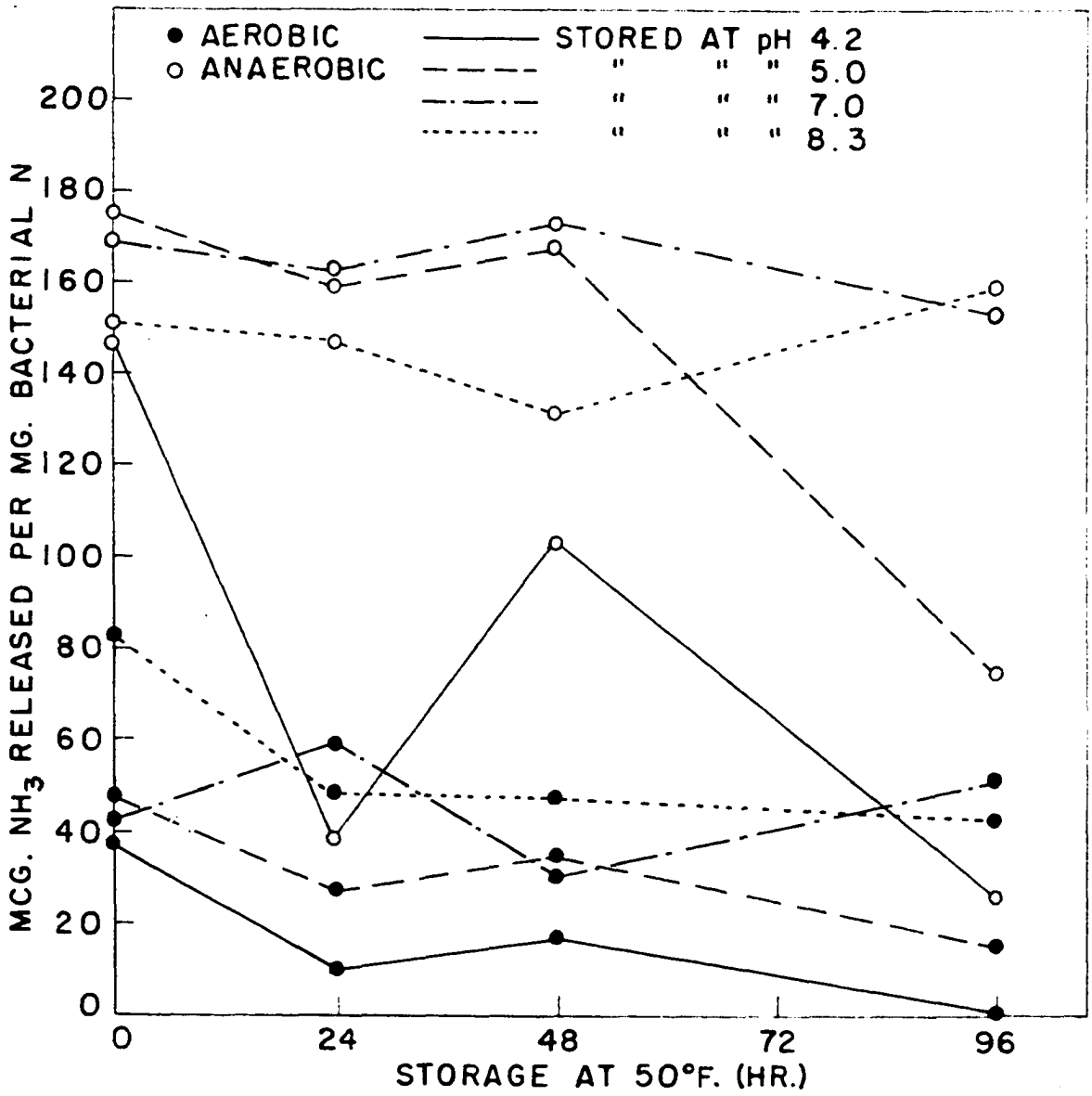

Figure 13. Effect of anaerobic-reduced storage conditions at different pH levels of cultures of L. casei strain 7 on the ability of the cells to deaminate DL-serine. Deamination at pH 8.3, 46° C. under aerobic and anaerobic-reduced conditions

conditions when such cells were tested at pH 5.4, 52° C. and pH 8.3, 46° C., respectively. Table 8 shows the changes in pH of the subcultures during the 96 hr. of storage.

The results show the initial adjustment of pH affected the deaminase activity of the cells considerably. Tested at pH 5.4 (Figures 10 or 12) none of the cells showed much activity under aerobic testing conditions. Under anaerobic-reduced deaminase test conditions the cells adjusted to pH 4.2 gave negative results, more NH_3 being consumed than was released. Cells adjusted to pH 7.0 showed some reduction in initial activity, compared to non-adjusted cells at pH 5.0, but were still quite active. Adjustment to pH 8.3 increased the initial deaminase activity of the cells about 100 per cent.

Tested at pH 8.3 (Figures 11 or 13) the non-adjusted cells tested under both aerobic and anaerobic-reduced conditions showed more activity than the adjusted cells at 0 hr., except for the cells which had been adjusted to pH 8.3 and then were tested under aerobic conditions.

Adjustment to pH 8.3 and storage under aerobic conditions seemed to preserve the enzyme activity of the cells best when the deaminase test was conducted at either pH 5.4 or pH 8.3 (Figures 10, 11, 12 and 13). Adjustment of the subculture to pH 4.2 seemed to be harmful to the enzyme. A few of the curves showed some fluctuation in deaminase activity during the 96 hr. period, particularly the curves representing

Table 8

Changes in pH during 96 hr. storage of cultures of *L. casei* strain 7 initially adjusted to pH 4.2, 5.0, 7.0 and 8.3

Initial pH	Storage time hr.	pH after storage under conditions which were:	
		Aerobic	Anaerobic-reduced
4.2	0	4.2	4.2
	24	4.21	4.21
	48	4.18	4.20
	96	4.20	4.20
5.0	0	5.0	5.0
	24	4.76	4.80
	48	4.59	4.78
	96	4.48	4.78
7.0	0	7.0	7.0
	24	6.58	6.48
	48	5.88	5.90
	96	5.21	5.38
8.3	0	8.30	8.30
	24	8.30	8.30
	48	8.15	8.08
	96	7.98	8.08

deamination at pH 8.3, but the general tendency was toward decreasing activity by 96 hr.

The figures in Table 8 show the pH did not remain constant during storage except in subcultures stored at pH 4.2. The cells from these subcultures showed the least fluctuation and the smallest values of deaminase activity compared to the other cells. Probably the changes in deaminase activity of subcultures stored at pH 4.2 only can be attributed to the increase in age of the cells. At pH 4.2, many enzymes would not be active and pH would not shift so much. The subcultures stored at the higher pH levels all showed a decrease of pH during storage. Consequently the change in deaminase activity could at least in part be due to a change in the physiological state of the cells (Figure 7) rather than to an increase in age alone.

The results of this experiment also show the previously demonstrated differences in the stimulatory effect of anaerobic-reduced deaminase conditions on cells in different physiological states (Figure 7). Except for the initial results of cells tested at pH 8.3 (Figure 11 or 13), anaerobic-reduced conditions stimulated the deaminase activity of cells stored at pH 5.0 or above comparatively more than of cells stored at pH 4.2. The high initial stimulation when the cells adjusted to pH 4.2 were tested at pH 8.3 could be because the adjustment of the medium to pH 4.2 had not fully affected the cells at this time.

Occurrence in Cheddar cheese of lactobacilli capable of producing hydrogen sulfide and deaminating serine

Isolations of lactobacilli were made from 14 samples of cheese to determine if the ability of L. casei strain 7 to produce H_2S and deaminate serine was an "isolated" case or if such types of bacteria occurred frequently in American Cheddar cheese without previously having been recognized. The cheese included some of both good and poor quality and that made from both raw and pasteurized milk.

The procedure and selective medium described under Experimental Methods were used for plating. Ten colonies of different appearance were picked from the plates of each cheese into tubes of the V-8 juice medium. After 24 hr. at 32° C. the cultures were examined under the microscope and the tubes containing rod-shaped organisms replated until pure cultures were assured.

The pure cultures were identified as being L. casei types on the basis of the following results: Gram positive short to medium, slender rods forming short to long chains in V-8 juice broth. Acid produced in litmus milk with reduction. Optimum temperature for growth 37° C. and growth obtained at 16° C. Sugar fermentation tests were not performed.

Some of the isolates from the same cheese were obviously duplicates and were discarded. V-8 juice cultures were grown

of the remainder at 32° C. for 16 hr. and then checked for pH and H_2S formation. The cells were harvested and examined for deaminase activity against DL-serine under aerobic and anaerobic-reduced conditions at pH 5.4 and pH 8.3. The results are listed in Table 9.

The table does not include isolates from cheese 4, 5 and 6, all very poor quality cheese. Lactobacilli were not found in cheese 4 and 5. The type found in cheese 6 could not be precipitated by centrifugation due to a layer of slime around the cells. (Most of the different strains appeared to be capsulated, when examined under the microscope, but little difficulty was encountered in harvesting except for the culture from cheese 6 and L. casei strain 142.)

L. casei strains 25, 28 and 142, which were tested for deaminase activity at 37° C. in the preliminary investigations, were tested at the higher temperatures and the results included in Table 9. Culture FS, the Streptococcus lactis starter which was to be used in the subsequent cheese making experiments, also was tested for serine deaminase activity at 37, 46 and 52° C. and the results included in Table 9.

All of the cultures, as mentioned above, were incubated at 32° C. for 16 hr. No attempt was made to harvest the different cultures at the same pH, since this would have complicated the study considerably. The previous results with L. casei strain 7, also had indicated that any ability to deaminate

Table 9

Production of H_2S and deamination of DL-serine by L. cheese, L. casei strains 25, 28 and 142 and S.

Cheese no. and milk type	Flavor score	Isolates	pH at harvest time	H_2S	Mg. bacterial N per tube
1. past.	39.0	1-1	4.28	0	1.76
		1-2	4.30	0	1.55
		1-3	4.30	0	1.13
		1-5	4.27	+	1.41
2. past.	37.5	2-1	4.25	0	1.34
3. past.	39.5	3-1	4.68	+	1.13
		3-3	4.12	0	1.34
7. past.	38.0	7-1	5.23	0	0.71
8. raw	40.0	8-1	4.55	+	0.91
		8-2	4.42	0	1.34
9. raw	37.0	9-1	4.02	0	1.27
		9-2	4.23	0	1.27
10. raw	38.5	10-4	4.31	0	1.06
		10-5	4.45	0	1.06
11. past.	38.5	11-7	4.80	0	0.92
12. past.	38.5	12-1	4.15	0	1.41
13. past.	40.0	13-1	4.20	+	1.63
14. past.	39.0	14-3	4.29	+	1.34
		14-4	4.31	0	1.41
-	-	25	4.61	0	1.20
-	-	28	4.38	0	1.06
raw	-	142	4.32	0	0.92
-	-	FS	4.38	0	1.10
-	-	FS-37° C.*	4.28	0	1.10

*Deamination at 37° C.

Table 9

i deamination of DL-serine by L. casei types isolated from
. strains 25, 28 and 142 and S. lactis culture FS

pH at harvest time	H_2S	Mg. bacterial N per tube	Mcg. NH_3 per mg. bacterial N at: pH 5.4, 52° C. Aerobic	pH 5.4, 52° C. Anaerobic-reduced	pH 8.3, 46° C. Aerobic	pH 8.3, 46° C. Anaerobic-reduced
4.28	0	1.76	8.7	9.3	70.3	82.8
4.30	0	1.55	29.7	35.5	32.4	36.6
4.30	0	1.13	7.6	6.7	11.8	13.1
4.27	+	1.41	7.1	7.4	36.3	49.2
4.25	0	1.34	0.8	3.4	6.6	3.0
4.68	+	1.13	17.1	26.8	53.6	148.2
4.12	0	1.34	16.0	28.0	13.3	18.4
5.23	0	0.71	3.8	9.5	20.1	27.7
4.55	+	0.91	10.2	24.0	60.8	55.8
4.42	0	1.34	3.2	10.7	73.6	92.5
4.02	0	1.27	5.8	12.2	12.6	21.4
4.23	0	1.27	18.7	34.9	25.7	37.4
4.31	0	1.06	12.4	10.0	6.6	5.0
4.45	0	1.06	2.7	26.3	95.4	146.0
4.80	0	0.92	-2.5	7.8	46.7	152.5
4.15	0	1.41	6.3	12.3	12.2	19.4
4.20	+	1.63	1.9	10.8	20.2	25.2
4.29	+	1.34	39.8	46.7	92.3	111.8
4.31	0	1.41	-0.1	3.0	10.2	11.8
4.61	0	1.20	0.0	-0.2	3.8	0.0
4.38	0	1.06	6.6	8.4	16.0	24.4
4.32	0	0.92	5.8	10.5	16.3	78.2
4.38	0	1.10	-1.8	-	0.0	6.4
4.28	0	1.10	-0.6	-	0.4	-

serine, which such types of lactobacilli might possess, probably would show under anaerobic-reduced deaminase conditions at any of the pH levels at which these cultures were harvested.

Five of the isolates from five different samples of cheese showed ability to produce H_2S in V-8 juice broth. The five samples of cheese which contained this type of L. casei all had a flavor score of 39.0 or better (Grade A cheese). Four of the samples were made from pasteurized milk, one from raw. None of the isolates from cheese with flavor scores below 39.0 produced H_2S. This does not necessarily mean this type of L. casei was not present in the poorer cheese, as the number of colonies picked was limited, but it could indicate the type is more numerous in good cheese.

The isolates showed a varied ability with regard to deamination of DL-serine. Some strains showed a deaminase activity comparable to that of L. casei strain 7, at both pH 5.4 and 8.3. Others had comparatively more activity in the acid range than in the alkaline. A few strains showed only little activity in both ranges. The strains of lactobacilli producing H_2S did not always show the most deaminase activity. In general, anaerobic-reduced conditions stimulated deamination of DL-serine by the isolates.

From these results it is difficult to say if the ability of L. casei to deaminate serine has a bearing on the quality of the cheese. Lactobacilli could not be isolated from some

of the poorest cheese and the isolates from other poor cheeses did not possess much deaminase activity. However, the latter was also the case with some of the isolates from good cheese, although the good cheese in general also contained some strains with a rather high activity. Again it must be pointed out that the number of cheese and isolates were comparatively few and the conditions under which deaminase activity was tested were not necessarily optimum for the different strains.

Culture 25 did not show any improvement in deaminase activity at the higher temperatures. Cultures 28 and 142 showed quite an increase over 37° C. The histories of cultures 25 and 28 are unknown. Culture 142 originally was isolated from raw milk cheese. Starter culture FS did not show much deaminase activity under any conditions.

Cheesemaking Experiments

Arrangement

Of the six series of cheese made, Series I and III were to study the effect of added serine with and without L. casei added. Two vats of cheese were made in each series, each out of 212 lb. of milk. L. casei strain 7 was added to one vat prior to setting. After milling, the curd in each vat was divided in two equal halves, and DL-serine added to one half in each vat, together with the salt, at the rate of 0.5 gm. of DL-serine per pound of curd. Salt only was added to the other half.

Series II and IV were to study the effect of whey globulin, which contains the milk phosphatases, and of potato phosphatase on cheese made from pasteurized milk with L. casei strain 7 added. Three vats of cheese were made in each series, each vat containing 155 lb. of milk. One vat served as control. Whey globulin from 12.6 l. of raw whey were added to the second vat. No attempt was made to add a specified amount of phosphatase, but the globulin preparations were checked and found to possess phosphatase activity at pH 5.4. The third vat had potato phosphatase preparation added. Raw milk and pasteurized milk from the same source were compared for phosphatase activity at pH 5.4. Enough potato phosphatase

preparation, the activity of which had been checked at pH 5.4, was added to the cheese milk to make up for the reduction due to pasteurization.

The last two series, V and VI, were made to investigate if the amount of potato phosphatase preparation added to the milk influenced the quality of the cheese. L. casei strain 7 was added to all the vats.

The cheese was checked for phosphatase activity at pH 5.4 and free amino acids on the second day after making. The examinations after 1, 3 and 6 months included phosphatase activity at pH 5.4, pH, numbers of lactobacilli, -SH groups and "free" H_2S, total-, soluble- and NH_3-nitrogen, free amino acids and quality. Series I and III were not checked for phosphatase activity.

For simplicity's sake only one table is made up for each type of analysis. This table contains the results from the entire 6 month period for all six series of cheese. The series are not listed chronologically but in pairs according to similarity.

Table 10 is a key to the cheese within each series. In the tables containing the results, the series will be referred to by number and the cheese within the series by letter.

Table 10

Key to the cheese within each series

Series no.	Cheese	Experimental
I	a	control
and	b	control + serine
III	c	control + L. casei
	d	control + L. casei + serine
II	a	control (L. casei added)
and	b	control + whey globulins
IV	c	control + potato phosphatase
V	a	control (L. casei added)
and	b	control + 1 X potato phosphatase
VI	c	control + 2 X potato phosphatase

Phosphatase activity at pH 5.4 of milk, whey and cheese

Samples of milk and whey were taken from each vat to check on the phosphatase activity. The milk sample was taken just prior to setting and the whey sample just before the whey was removed from the curd. The cheese was checked for phosphatase activity at 2 days and at 1, 3 and 6 months. The results are listed in Table 11.

The results show phosphatase activity at pH 5.4 of milk was reduced approximately 40-50 per cent by pasteurization. Addition of whey globulins decreased the phosphatase activity slightly, although the globulins had been demonstrated to have

Table 11

Phosphatase activity at pH 5.4 of milk, whey and cheese

Series	Cheese	Mcg. phenol released per ml. of:			Mcg. phenol released per gm. of cheese at:			
		Raw milk	Past. milk	Whey	2 days	1 month	3 months	6 months
II	a		13.1	2.4	71.3	76.3	77.5	78.8
	b	23.3	10.4	2.9	78.8	92.5	78.8	63.8
	c		20.1	10.9	81.3	110.0	76.3	50.0
IV	a		12.5	5.9	111.3	95.0	118.5	79.5
	b	21.8	10.3	6.0	91.3	91.3	110.0	67.5
	c		20.5	15.1	88.8	86.3	108.5	63.3
V	a		16.8	8.0	97.5	90.0	100.0	63.8
	b	26.8	24.9	16.8	100.0	77.5	101.0	56.3
	c		31.5	27.9	80.0	87.5	104.0	46.3
VI	a		11.8	7.5	83.8	87.5	107.5	80.0
	b	23.5	17.9	11.4	88.8	96.3	100.0	62.5
	c		22.4	17.3	86.8	83.8	107.0	65.0

some phosphatase activity. Addition of potato phosphatase preparation increased the phosphatase activity of the pasteurized milk to approximately the range of raw milk. However, Series V-c, with twice the amount, and Series VI-b and c, with both normal and twice the amount of potato phosphatase preparation added, showed much smaller increases in activity in the milk than was calculated.

The control whey and the whey from the milk with whey globulin added showed about the same amount of activity. The whey from milk with potato phosphatase preparation added showed a much higher activity than the control and this difference was greater when the larger amounts of potato phosphatase preparation were added. Laboratory experiments conducted with raw milk indicated the additional phosphatase activity at pH 5.4 of such milk also was lost with the whey.

Determination of phosphatase activity at pH 5.4 of the cheese indicated that acid phosphatase was retained in the cheese in varying amounts apparently independent of the original amount in the milk. The phosphatase activity of the cheese fluctuated somewhat between 2 days and 3 months. The determinations at 6 months showed a decrease in activity of all the cheese except cheese a in Series II. The cheese with added phosphatase preparations, particularly with added potato phosphatase preparation, showed a larger decrease than the control cheese. Since all four series of cheese contained added L.

casei, no data are available on the influence of this organism on phosphatase activity.

Changes in pH during ripening

The pH of the cheese was only measured at 3 and 6 months. Table 12 shows the results. In no one series at any one sampling period did the differences within the series exceed 0.1 pH unit; frequently the differences were much smaller, so the trends within a series at one sampling are at best suggestive because the values actually all are within the commonly accepted limits of error of the method of measurement.

The increase in pH during the 6 months ripening period were not as large as anticipated. Cheese without L. casei added showed the most increase in pH from 3 to 6 months and the highest pH values at 6 months. Addition of serine could possibly be the reason for the slightly higher pH values of cheese b and c in Series I and III. Addition of phosphatase preparations did not result in a definite trend. The lower pH of cheese with L. casei added was probably due to increased bacterial activity with more acid having been produced in this cheese either initially from the lactose or during ripening, possibly by deamination.

Table 12

Changes in pH during ripening

Series	Cheese	pH at: 3 months	pH at: 6 months
I	a	5.03	5.20
	b	5.07	5.25
	c	5.05	5.17
	d	5.04	5.18
III	a	5.00	5.20
	b	5.02	5.24
	c	4.98	5.12
	d	5.02	5.16
II	a	4.98	4.99
	b	4.95	5.03
	c	5.00	5.09
IV	a	4.92	5.04
	b	4.97	5.09
	c	5.01	5.07
V	a	4.98	5.10
	b	4.95	5.07
	c	4.96	5.08
VI	a	4.95	5.11
	b	4.98	5.07
	c	4.99	5.05

Bacteriological examination

By using the selective medium mentioned under Experimental Methods it was found possible to follow the changes in the numbers of lactobacilli. The results are listed in Table 13. The uninoculated cheese did not contain many lactobacilli. The cheese without serine added showed an increase in lactobacilli during the 6 months period in both Series I and II. The cheese with serine added in Series I contained a much higher number of lactobacilli at the 1 and 3 months examination. Although the lactobacilli increased in numbers in the control cheese up to 6 months, they disappeared in the cheese with serine added. Series III showed a slightly different picture, the growth of lactobacilli being somewhat delayed in the cheese with serine added and the number of lactobacilli in this cheese not exceeding the number of lactobacilli in the control cheese until the examination at 6 month.

The numbers of lactobacilli in all of the inoculated cheese were high. Probably because of the high initial inoculation, neither addition of serine or of phosphatase preparations seemed to have any effect on the numbers. The tendency in all the inoculated cheese seemed to be a slight decrease in numbers of lactobacilli from 1 to 3 months and a rather large decrease from 3 to 6 months.

Table 13

Lactobacillus counts of experimental cheese

Series	Cheese	Counts (millions per gm. of cheese) at: 1 month	3 months	6 months
I	a	0.00005	0.0025	0.037
	b	0.0025	0.125	0.00005
	c	110	120	15
	d	96	110	20
III	a	0.00065	0.35	0.49
	b	0.00075	0.08	0.82
	c	280	190	62
	d	260	220	77
II	a	170	120	7.5
	b	52	53	20
	c	280	170	4
IV	a	230	230	53
	b	290	130	22
	c	370	140	23
V	a	590	450	110
	b	330	390	160
	c	660	410	90
VI	a	600	350	190
	b	580	240	220
	c	460	440	140

Sulphhydryl groups and "free" hydrogen sulphide

Sulphhydryl groups and H_2S have been reported to be present in all New Zealand Cheddar cheese, as mentioned in the review of literature. Lactobacilli were postulated to cause the release of H_2S, but direct proof was never offered. Since L. casei strain 7 and other L. casei types had been found to produce H_2S in V-8 juice broth, the nitroprusside test was included for the examination of the cheese to detect -SH groups and H_2S. However, it was also found that by boiling mildly acidified cheese suspensions and letting the vapor pass through a piece of filter paper wetted with lead acetate solution, the H_2S present in the cheese was detectable. This method was used to determine the relative amounts of "free" H_2S in the experimental Cheddar cheese. The results are recorded in Table 14.

The amounts of -SH groups and "free" H_2S increased as the cheese ripened. The increase in "free" H_2S is not always apparent from the table. The results as recorded are relative within the series and fairly much within an examination period. They should not be compared from period to period. Irregardless of the number of plusses given a cheese from period to period, an increase in "free" H_2S took place with ripening. The occasional decrease in the number of plusses from one period to the next for some cheese was due to a shift in intensity

Table 14

Micrograms of -SH groups and relative intensity of "free" H_2S

Series	Cheese	1 month		3 months		6 months	
		Mcg. of -SH per gm. of cheese	Relative intensity of H_2S	Mcg. of -SH per gm. of cheese	Relative intensity of H_2S	Mcg. of -SH per gm. of cheese	Relative intensity of H_2S
I	a	5	+	5-10	++	10-15	+(+)
	b	5	+(+)	5-10	+(+)	10+	+++
	c	5-10	++	10-15	+++	15-20	+++(+)
	d	5-10	++	10	++(+)	20	++++
III	a	5	+	5-10	++	10-15	+
	b	5	+(+)	5-10	++(+)	15	++
	c	5-10	++	10-15	+++	15	++(+)
	d	5-10	++	10-15	+++	15-20	++
II	a	5	+	5-10	+	20-25	++
	b	5	+(+)	10-15	+++	25+	++++
	c	5+	++	10-15	++	25	+++
IV	a	5-10	++	10-15	++	20	+(+)
	b	5-10	++	15-20	++++	25	++++
	c	5-10	++(+)	10-15	+++	25	+++(+)
V	a	5	+	10-15	+++	15-20	++
	b	5	+(+)	5-10	++	10-15	+++
	c	5	++	10-15	+++	25-30	+++
VI	a	5	+	10	++	20	++
	b	5	+(+)	10-15	+++	20-25	++++
	c	5	+(+)	10	++	10-15	+

compared to the other cheese in the same series.

The concentration of -SH groups and the relative concentration of "free" H_2S corresponded well at the 1 month examination. As the cheese got older, and particularly at 6 months, some variation was apparent in this relationship. Some cheese with lower concentrations of -SH groups were found to contain more "free" H_2S than cheese in the same series with higher concentrations of -SH groups. Other cheese contained comparatively much more "free" H_2S than cheese in the same series with a similar or slightly lower -SH group content.

Cheese without L. casei added (Series I and III, cheese a and b) contained fewer -SH groups and less "free" H_2S than inoculated cheese. The difference increased with ripening. Addition of serine to such cheese (Series I and III, cheese b) caused an increase in -SH groups and particularly in "free" H_2S. The difference between inoculated cheese with and without serine added was very slight (Series I and III, cheese c and d).

Addition of milk phosphatase preparation always resulted in an increase in -SH groups and "free" H_2S. The production of H_2S especially was much higher in such cheese than in the control (Series II and IV, cheese b). Potato phosphatase preparations showed the same effect in general. Although the release of H_2S did not quite compare to that obtained with

added milk phosphatase preparations, it was higher than the corresponding control. Increased amounts of potato phosphatase preparations seemed to have varying effects.

Protein breakdown

The changes in the soluble nitrogen and NH_3 nitrogen fractions were followed and reported as per cent of the cheese and per cent of total nitrogen. The soluble nitrogen was the nitrogen not precipitated by addition of 0.2 N CH_3COOH to bring the cheese solution to pH 4.6-4.7. The results are recorded in Table 15. The figures show an increase in both soluble nitrogen and NH_3 nitrogen as the cheese matured. However, there was no apparent correlation between increases in these fractions and addition of L. casei, serine, whey globulins or potato phosphatase preparation.

Free amino acids

Appearance and increase in concentration of the free amino acids in the cheese was followed by the paper chromatographic method outlined under Experimental Methods. Some overlapping of the amino acid spots occurred in certain areas, notably between aspartic and glutamic acids, asparagine and glutamine, and leucine and isoleucine. This probably was due in part to

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Series	Cheese	1 month					
		In % of cheese			In % of Total N		In %
		Total N	Sol. N	NH_3-N	Sol. N	NH_3-N	Total N
I	a	3.91	0.54	0.034	13.8	0.87	4.01
	b	3.94	0.53	0.036	13.5	0.91	4.01
	c	3.96	0.50	0.035	12.6	0.88	4.01
	d	3.98	0.52	0.035	13.1	0.88	3.98
III	a	3.81	0.52	0.042	13.6	1.10	3.92
	b	3.74	0.52	0.044	13.9	1.18	3.88
	c	3.67	0.52	0.048	14.4	1.31	3.85
	d	3.83	0.52	0.050	13.6	1.31	3.94
II	a	3.71	0.58	0.037	15.6	1.00	3.94
	b	3.70	0.54	0.035	14.6	0.95	3.87
	c	3.70	0.50	0.028	13.5	0.76	3.85
IV	a	3.49	0.50	0.039	14.3	1.12	3.61
	b	3.89	0.63	0.048	16.2	1.23	4.00
	c	3.93	0.51	0.049	13.0	1.12	3.95
V	a	3.78	0.50	0.046	13.2	1.22	3.80
	b	3.75	0.47	0.046	12.5	1.23	3.76
	c	3.77	0.47	0.044	12.5	1.17	3.76
VI	a	3.80	0.52	0.035	13.7	0.92	3.83
	b	3.81	0.54	0.034	14.2	0.90	3.79
	c	3.77	0.49	0.035	13.0	0.92	3.81

Table 15

Protein breakdown

l N	3 months					6 months				
N	In % of cheese			In % of Total N		In % of cheese			In % of To	
	Total N	Sol. N	NH_3-N	Sol. N	NH_3-N	Total N	Sol. N	NH_3-N	Sol. N	NI
7	4.01	0.80	0.048	20.0	1.20	4.08	1.04	0.071	25.5	1
L	4.01	0.80	0.058	20.0	1.45	4.03	1.02	0.052	25.3	1
8	4.01	0.78	0.042	19.5	1.05	4.04	1.04	0.053	25.7	1
8	3.98	0.79	0.056	19.8	1.41	4.00	1.01	0.069	25.3	1
0	3.92	0.72	0.058	18.4	1.48	4.07	1.04	0.092	25.6	2
8	3.88	0.71	0.055	18.3	1.42	3.99	1.11	0.100	27.8	2
L	3.85	0.74	0.048	19.2	1.25	3.95	1.09	0.100	27.6	2
L	3.94	0.73	0.058	18.5	1.47	3.95	1.10	0.096	27.8	2
0	3.94	0.83	0.070	21.1	1.78	3.94	1.19	0.084	30.2	2
5	3.87	0.87	0.072	22.5	1.86	3.89	1.16	0.060	29.8	1
6	3.85	0.81	0.076	21.0	1.97	3.93	1.18	0.082	30.0	2
2	3.61	0.75	0.049	20.8	1.36	3.62	1.04	0.076	28.7	2
3	4.00	0.86	0.065	21.5	1.63	4.03	1.27	0.085	31.5	2
2	3.95	0.81	0.060	20.5	1.50	3.96	1.14	0.085	28.8	2
2	3.80	0.75	0.049	19.7	1.29	3.86	1.10	0.064	28.5	1
3	3.76	0.77	0.042	20.5	1.12	3.84	1.04	0.059	27.1	1
7	3.76	0.75	0.039	19.9	1.04	3.81	1.07	0.055	28.1	1
2	3.83	0.80	0.042	20.9	1.10	3.89	1.15	0.071	29.6	1
0	3.79	0.76	0.039	20.0	1.03	3.83	1.08	0.076	28.2	1
2	3.81	0.79	0.046	20.7	1.21	3.81	1.09	0.075	28.6	1

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n breakdown

3 months				6 months				
of cheese		In % of Total N		In % of cheese			In % of Total N	
Sol. N	NH_3-N	Sol. N	NH_3-N	Total N	Sol. N	NH_3-N	Sol. N	NH_3-N
0.80	0.048	20.0	1.20	4.08	1.04	0.071	25.5	1.74
0.80	0.058	20.0	1.45	4.03	1.02	0.052	25.3	1.29
0.78	0.042	19.5	1.05	4.04	1.04	0.053	25.7	1.31
0.79	0.056	19.8	1.41	4.00	1.01	0.069	25.3	1.73
0.72	0.058	18.4	1.48	4.07	1.04	0.092	25.6	2.26
0.71	0.055	18.3	1.42	3.99	1.11	0.100	27.8	2.51
0.74	0.048	19.2	1.25	3.95	1.09	0.100	27.6	2.53
0.73	0.058	18.5	1.47	3.95	1.10	0.096	27.8	2.43
0.83	0.070	21.1	1.78	3.94	1.19	0.084	30.2	2.13
0.87	0.072	22.5	1.86	3.89	1.16	0.060	29.8	1.54
0.81	0.076	21.0	1.97	3.93	1.18	0.082	30.0	2.09
0.75	0.049	20.8	1.36	3.62	1.04	0.076	28.7	2.10
0.86	0.065	21.5	1.63	4.03	1.27	0.085	31.5	2.11
0.81	0.060	20.5	1.50	3.96	1.14	0.085	28.8	2.15
0.75	0.049	19.7	1.29	3.86	1.10	0.064	28.5	1.66
0.77	0.042	20.5	1.12	3.84	1.04	0.059	27.1	1.54
0.75	0.039	19.9	1.04	3.81	1.07	0.055	28.1	1.44
0.80	0.042	20.9	1.10	3.89	1.15	0.071	29.6	1.83
0.76	0.039	20.0	1.03	3.83	1.08	0.076	28.2	1.98
0.79	0.046	20.7	1.21	3.81	1.09	0.075	28.6	1.97

the small size of the filter paper in proportion to the large application and relatively high concentration of these amino acids and amides. The two former pairs could be separated with fairly good accuracy because of the different colors of the developed spots, but leucine and isoleucine were difficult to separate and are recorded as one value. The results recorded in Table 16 are relative with regard to intensity and size of the spots of the individual amino acids.

The free amino acid patterns were not much different from the results obtained by other investigators. A few exceptions were noted in the number of amino acids. Alanine did not appear except in a few cases at the 1 month examination. Threonine and methionine, which also have been reported present in small amounts by other investigators, did not appear either.

Serine, which was of principal interest in this study, appeared in strong concentration at the 2 day examination in the cheese to which DL-serine had been added (cheese b and d of Series I and III). It was not apparent in the control cheese of those series. At 1 month the concentration of serine appeared to have decreased in this cheese, most in the cheese with L. casei added. Serine still did not appear in the control cheese. The 3 months examination of Series I revealed presence of serine only in cheese b. However, serine was present in Series III in all four cheeses, with a higher

Appearance and relative concentration of

Amino acids	Series I				Series III				
	a	b	c	d	a	b	c	d	a
Aspartic acid	(+)*	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(
Glutamic acid	+	+	+	+	+	+	+	+	+
Serine	-	++++	-	++++	-	++++	-	++++	-
Lysine	+	+	+	+	+	+	+	+	+
Valine	-	-	-	-	-	-	-	-	-
Aspartic acid	(+)	(+)	+	+	(+)	(+)	+	+	(
Glutamic acid	+	+	++	++	+	+(+)	++	++(+)	+
Serine	-	++	-	+	-	++	-	+	-
Alanine	-	-	-	-	-	+	+	+	-
Lysine	+	+	+	+	+	+	++	++	+
Tyrosine	-	-	-	-	-	-	-	-	-
Valine	-	-	-	-	-	trace	trace	trace	(
Leucines	-	+	++	++	+	+	++	++(+)	+
Phenylalanine	+	+	++	+(+)	-	+	++	++	-
Aspartic acid	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(
Glutamic acid	+++	++	++	++	++++	+++	+++	+++	+
Serine	-	+	-	-	+	++	+	+	-
Glycine	-	-	-	-	-	-	-	-	+
Asparagine	++	+	+	++	++	(+)	(+)	(+)	+
Lysine	++	+	+	(+)	++	(+)	(+)	(+)	+
Arginine	+	(+)	(+)	-	+	(+)	(+)	-	+

Table 16

Concentration of free amino acids as determined chromatographically

		Series II			Series IV			Series V			S
c	d	a	b	c	a	b	c	a	b	c	a
			2 days								
(+)	(+)	(+)	(+)	(+)	(+)	(+)	+	(+)	+	+	-
+	+	+	+	+	+	+	++	+	++	++	+
-	++++	-	-	-	-	-	-	-	-	-	-
+	+	+	+	+	+	+	++	+	+	+(+)	+
-	-	-	-	-	trace	trace	trace	trace	trace	trace	trac
			1 month								
+	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	-	-	-
++	++(+)	++	++	++	+(+)	++	+(+)	++	+	++	++
-	+	-	-	-	-	-	-	-	-	(+)	-
+	+	-	-	-	-	-	+	-	-	-	-
++	++	+	+	+	+	+(+)	+(+)	+	(+)	+	+
-	-	-	-	-	-	-	-	trace	trace	trace	trac
trace	trace	(+)	-	(+)	(+)	(+)	(+)	+	(+)	+	+
++	++(+)	++	+(+)	+(+)	+	++(+)	++	++	+	++	++
++	++	-	-	-	+	++(+)	++	++	+	++	++
			3 months								
(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
+++	+++	+++	++	++	+++	+	++	+++	+	++	+++
+	+	-	+(+)	++	+	-	+	++	(+)	+++	(+)
-	-	++	-	+	-	-	-	-	-	-	-
(+)	(+)	++	+	+	+	(+)	+	+	(+)	+	+
(+)	(+)	++	+	+(+)	+	-	(+)	+	+	+	+
(+)	-	++	+	+	-	-	-	-	-	-	(+)

.e 16

amino acids as determined chromatographically

ies II		Series IV			Series V			Series VI		
b	c	a	b	c	a	b	c	a	b	c
lays										
(+)	(+)	(+)	(+)	+	(+)	+	+	-	+(+)	+
+	+	+	+	++	+	++	++	+	++	++
-	-	-	-	-	-	-	-	-	-	-
+	+	+	+	++	+	+	+(+)	+	+(+)	+
-	-	trace	trace	trace	trace	trace	trace	trace	trace	trace
ıonth										
(+)	(+)	(+)	(+)	(+)	(+)	-	-	-	-	-
++	++	+(+)	++	+(+)	++	+	++	++	+	++
-	-	-	-	-	-	-	(+)	-	-	-
-	-	-	-	+	-	-	-	-	-	-
+	+	+	+(+)	+(+)	+	(+)	+	+	(+)	+
-	-	-	-	-	trace	trace	trace	trace	trace	trace
-	(+)	(+)	(+)	(+)	+	(+)	+	+	+	+
+(+)	+(+)	+	++(+)	++	++	+	++	++	+(+)	++
-	-	+	++(+)	++	++	+	++	++	+(+)	++
ıonths										
(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
++	++	+++	+	++	+++	+	++	+++	++	+++
+(+)	++	+	-	+	++	(+)	+++	(+)	+	(+)
-	+	-	-	-	-	-	-	-	-	-
+	+	+	(+)	+	+	(+)	+	+	+	++
+	+(+)	+	-	(+)	+	+	+	+	+	+
+	+	-	-	-	-	-	-	(+)	(+)	-

Tab.

Amino acids	Series I				Series III			
	a	b	c	d	a	b	c	d
Tyrosine	-	-	-	-	(+)	(+)	(+)	-
Valine	+++	(+)	+++	+	++	+	(+)	(+)
Leucines	++++	++	+++	++	++++	++	++	+++
Phenylalanine	++++	(+)	++	(+)	++++	+	++	+++
Gamma amino-butyric acid	-	-	-	-	-	-	-	-
Aspartic acid	+	+(+)	+(+)	(+)	(+)	+(+)	+(+)	+
Glutamic acid	+++	++++	++++	+	+	+++	+++	+++
Serine	++	+	+	-	-	+	-	trace
Glycine	++	++	++	+	+	++	+++	++
Asparagine	+++	+	+++	+	-	-	++	+
Glutamine	++	+	-	-	(+)	(+)	++	++
Lysine	+++	+	-	(+)	++	(+)	+	++
Histidine	+	+	+	-	-	-	+	(+)
Arginine	+	+	+	-	-	-	++	-
Tyrosine	+	-	(+)	-	-	-	-	-
Valine	+	-	+	-	-	-	-	-
Leucines	+++	+++	+++	(+)	+	+++	+++	++
Phenylalanine	+	-	+	-	(+)	+	++	(+)
Gamma amino-butyric acid	-	-	(+)	-	(+)	-	(+)	(+)

*Parenthesis means half value.

Table 16 (continued)

		Series II			Series IV			Series V			Se
c	d	a	b	c	a	b	c	a	b	c	a
			3 months (continued)								
(+)	-	+	+	+	(+)	(+)	(+)	(+)	+	+	+
(+)	(+)	+++	++	+	+(+)	-	+	++	+	+(+)	+
++	+++	++++	+++	++(+)	++	+	++	++++	++	++++	+++
++	+++	+++	++(+)	++	+++	+	++	++	+	++	+
-	-	(+)	-	(+)	-	-	-	-	-	-	-
			6 months								
+(+)	+	+	+	+(+)	+	+(+)	+	+(+)	+	+	(+)
+++	+++	++	+	+++	++	+++	+++	+++	+++	+++	++
-	trace	++	+	++	-	(+)	-	++	(+)	(+)	(+)
+++	++	+++	+++	+++	++	++	+	+++	++	+	-
++	+	+	++	+++	+	++	+	+++	+++	+(+)	++
++	++	+	+	+	-	++	(+)	++	-	-	-
+	++	-	+	++	++	+++	+	+	+	+	+
+	(+)	-	-	-	-	-	-	-	-	-	-
++	-	(+)	(+)	+	++	++	++	+	+	++	-
-	-	(+)	+	+	(+)	(+)	+	++	+	(+)	(+)
-	-	+	++	+++	(+)	+++	++	+(+)	++	+	+(+)
+++	++	++++	++(+)	++++	++	++++	++++	+++	++++	+++	+++
++	(+)	++	++	+++	+	+++	++	++++	+++	++	+
(+)	(+)	+	+	+	-	+	+	+	+	+	+

:ontinued)

.es II		Series IV			Series V			Series VI		
b	c	a	b	c	a	b	c	a	b	c
onths (continued)										
+	+	(+)	(+)	(+)	(+)	+	+	+	(+)	+
++	+	+(+)	-	+	++	+	+(+)	+	+	+
+++	++(+)	++	+	++	++++	++	++++	+++	+++	+++
++(+)	++	+++	+	++	++	+	++	+	+	+
-	(+)	-	-	-	-	-	-	-	+	+
onths										
+	+(+)	+	+(+)	+	+(+)	+	+	(+)	+	+
+	+++	++	+++	+++	+++	+++	+++	++	++	++
+	++	-	(+)	-	++	(+)	(+)	(+)	+	(+)
+++	+++	++	++	+	+++	++	+	-	(+)	-
++	+++	+	++	+	+++	+++	+(+)	++	+	++
+	+	-	++	(+)	++	-	-	-	+	+
+	++	++	+++	+	+	+	+	+	++	+
-	-	-	-	-	-	-	-	-	-	-
(+)	+	++	++	++	+	+	++	-	+	(+)
+	+	(+)	(+)	+	++	+	(+)	(+)	+	(+)
++	+++	(+)	+++	++	+(+)	++	+	+(+)	+	+
++(+)	++++	++	++++	++++	+++	++++	+++	+++	++++	++++
++	+++	+	+++	++	++++	+++	++	+	+++	+++
+	+	-	+	+	+	+	+	+	+(+)	+

concentration in cheese b, indicating release of more serine or less utilization than in Series I. At 6 months, serine was present in all the cheese in Series I, except d. On the other hand, serine had disappeared almost entirely from Series III.

Of the series with milk globulins and potato phosphatase preparations added, serine appeared at 1 month only in cheese c of Series V. The milk from which this cheese was made had showed the highest phosphatase activity of any (Table 11). At 3 months the serine pattern became somewhat uncertain. Serine appeared in most of the cheese, but no correlation between serine concentration and amount of phosphatase preparations added was apparent. The largest amount of serine was present in cheese c of Series V, which was the only cheese to show serine at 1 month.

The examination at 6 months revealed serine was now present in the cheese from which it was absent after 3 months of ripening. The cheese in which serine had been present at 3 months showed either a maintained concentration of serine or, in most cases, a relatively large decrease.

The other amino acids in general showed a rather uniform increase in concentration as ripening progressed, once they had appeared. The most notable exceptions were apparent in Series I and III, where phenylalanine and valine decreased in concentration between the 3rd and 6th months. The control

cheeses of the other four series showed the same tendency.

Flavor score

Table 17 illustrates the flavor score of the cheese as judged by two impartial, experienced judges. Additions of DL-serine in Series I resulted in inferior cheese when judged at 1 and 3 months on a numerical basis, but the cheese with serine added showed a higher intensity of Cheddar flavor at 3 months. At 6 months the cheese with DL-serine added showed quite an improvement in flavor score and was found better than the control cheese, which had decreased in quality. The cheese without L. casei added was found better than the cheese with L. casei added.

The cheese in Series III with DL-serine added did not show the same increase in flavor at 6 months; on the contrary, it scored lower than the control cheese. The cheese with DL-serine and without L. casei added was of good quality for 3 months and then developed inferior flavors. The cheese with both DL-serine and L. casei added was of poor initial quality, but improved with age, although never quite equalling its control.

Cheese with phosphatase preparations added all scored lower than the control cheese at 1 month, except for Series

Table 17

Flavor score and criticisms* of cheese at 1, 3 and 6 months

Series	Cheese	1 month	3 months		6 months	
		Flavor score and criticism	Flavor score and criticism	Intensity	Flavor score and criticism	Intensity
I	a	39.5 sl a	39.0 f, sl a	++	38.5 a, sl b	++
	b	38.5 f, sl b	38.5 a	+++	39.5	+++
	c	39.5 sl a	39.5 sl a	++	38.5 a, sl b	++
	d	39.0 sl a	38.0 a	+++	39.0 sl a	+++
III	a	38.5 f,a,sl b	39.5 a	+++	38.5 a, b, sl f	++
	b	39.0 f	39.0 sl a	++	38.0 f, b	++
	c	38.5 a	38.5 a, b	+++	39.0 a	+++
	d	38.0 a, b	38.25 a, sl b	++	38.75 a, sl b	++
II	a	39.5 f	38.5 a, sl b	++	38.0 a, m	+
	b	39.0 sl b, a	38.5 sl a, sl ferm	+	38.5 a, b	+++
	c	38.5 sl b, a	39.0 sl a	+++	39.5 sl a	+++
IV	a	38.0 a, b	38.5 b	++	38.0 b	+
	b	38.5 a, b	38.0 b, ferm	+	38.5 b, a	++++
	c	39.0 f, a	39.0 sl b, sl a	+++	38.5 a, sl b	+++
V	a	39.0 sl a	39.0 sl a, b	+++	38.5 a, b	+
	b	38.5 sl ferm	38.5 b, ferm	++	38.75 a, b	++
	c	38.25 sl a, b	38.75 sl a, b	++(+)	39.0 b	+++
VI	a	39.5 sl b	38.5 sl a, b	++	38.0 a, b	+
	b	39.0 sl a	39.0 sl a	+++	39.0 a	+++
	c	38.5 sl a, b	38.5 sl b, a	++(+)	38.5 a, b	+

*sl = slightly, a = acid, b = bitter, f = flat, ferm = fermented, m = musty.

IV, which had a comparatively poor control cheese. After 3 months the differences became less pronounced. The cheese with whey globulins added were still inferior to the control numerically and in flavor intensity, whereas the cheese with potato phosphatase preparations added on an average equalled the control cheese numerically and showed a slightly higher flavor intensity. At 6 months the cheese with either whey globulins or potato phosphatase preparations had improved in flavor and was found superior to the control cheese, which had decreased in flavor. Cheese with potato phosphatase preparations added was slightly better than cheese with whey globulins added. The higher level of potato phosphatase preparation appeared to be beneficial in Series V but the opposite was the case in Series VI.

Acid and bitter in varying degrees were the criticisms most often applied to the cheese.

DISCUSSION

The results demonstrate the ability of Lactobacillus casei strain 7 to release NH_3 in appreciable amounts from serine, cysteine and asparagine. The enzymes involved were probably those referred to as serine dehydrase, cysteine desulfarase and asparaginase, respectively. The reactions catalyzed by these enzymes are similar to the extent that apparently oxygen is not involved. Replacement of the hydroxyl hydrogen of serine with a phosphoric acid group resulted in inhibition of deamination of serine, which was not unexpected in view of the results of Chargaff and Sprinson (1943b) with Escherichia coli. The apparent lack of deaminase activity against the other amino acids tested is not taken as an indication that these amino acids are not important for growth of L. casei in Cheddar cheese. Many of these amino acids have been demonstrated to be essential for growth of L. casei in laboratory media, probably as building blocks, and very likely this would also apply in Cheddar cheese. The possibility of some of them being decarboxylated also exists. The balance of amino acids in the medium also has been demonstrated to be important for maximum growth of L. casei, a point which will be discussed later in this section.

The optimum pH for bacterial deaminase reactions has so far been considered to be about pH 8. The only exception known

is the deamination of cysteine by E. coli, which has an optimum of pH 6.4, as shown by Desnuelle and Fromageot (1939). The idea in this study of checking possible deaminase activity of L. casei at both pH 8.3 and 5.4 was meant only to be a routine comparison of deaminase activity at the respective pH levels. As it turned out, this dual checking of deaminase activity led to the discovery of the possible existence of two serine deaminases in certain strains of L. casei. One activity was at a maximum at pH 8.3, 46° C. and the other at pH 7, 52° C. both showing about the same level of maximum activity. A close relationship between the two enzyme systems was evident because the factors influencing production of enzyme affected both systems in the same manner. The possibility therefore exists that the results of the pH study were just a manifestation of the same enzyme with the balance between action-inactivation merely shifted by temperature. The pH study was the only one in which the reaction of both enzyme systems was carried out at optimum pH. In the other studies the reactions of the enzyme with an optimum at pH 7 were carried out at pH 5.4. However, if only one enzyme system was involved, it could be expected that the ratios of NH_3 released at pH 8.3, 46° C. to NH_3 released at pH 5.4, 52° C. would remain constant under similar conditions. This was not the case, as illustrated in Figure 7. A sizeable decrease in the ratio of the activity of the enzyme reacting

in the alkaline range to the one reacting in the acid range, with increasing physiological age, is evident under both aerobic and anaerobic-reduced deaminase conditions. Figure 7 also illustrates the comparatively larger response of the enzyme reacting at pH 5.4, 52° C. to anaerobic-reduced deaminase conditions, a difference which also was encountered in other experiments. These results could lend some support to the possible existence of two separate serine deaminases, but probably work with purified enzyme systems will have to be carried out before a final decision can be reached.

The optimum pH for the cysteine dehydrase or the asparaginase of L. casei strain 7 was not determined, but the results could indicate that the cysteine dehydrase had its optimum in the acid range like the one present in E. coli. The asparaginase definitely had its optimum in the alkaline pH range.

The failure of other investigators to find any appreciable deaminase activity by lactobacilli could be due to the use of lower incubation temperatures than those which in this study were found to be optima for deamination of serine. Incubation at 37° C. did not give much indication of deaminase activity, a fact which was only fully shown when the incubation temperature was increased considerably. The apparently slow rate of deamination at 37° C. could be due to an about equal consumption of NH_3 at this temperature. The increase

in the rate of deamination with increasing temperatures could possibly in part be because the activity of the enzymes involved in utilization of NH_3 did not increase at a rate equal to that of the deaminases or that these enzymes were inhibited by the higher temperatures. Consumption of NH_3 by lactic acid bacteria was pointed out by Peterson et al. (1928), who found NH_3 production was small in cultures of such bacteria and often did not equal its consumption. That considerable degradation of cysteine and probably also of serine and asparagine does take place at regular growth temperatures was indicated by the appearance of considerable amounts of H_2S when growth was at 32° C., although the deamination studies showed release of only small amounts of NH_3 at 32° C.

The decreases in formation of H_2S in the medium and in the serine deaminase activity of cells grown in the presence of glucose could indicate that the enzymes involved are adaptive, a theory commonly advanced for bacterial deaminases. The decrease in activity of the two enzymes probably was due to competitive inhibition by glucose. Brinkley (1943) found desulfarase activity of cell-free extracts of E. coli was inhibited by addition of either glucose or serine. The inhibition was considered competitive because it disappeared when the added glucose or serine had been used up. He did not determine whether competitive inhibition was exhibited by glucose on the serine dehydrase activity of the extract, but

the present study could indicate this to be the case. A competitive inhibition of serine on cysteine desulfarase activity could possibly be the reason for the lower concentration of H_2S in some of the cheese. It is interesting to note that the cheese of Series III in which serine apparently was continued to be utilized between the third and sixth months showed a lower concentration of "free" H_2S at 6 months than the cheese of Series I in which utilization of serine apparently ceased between the third and sixth month. A difference in availability and utilization of serine could also be the reason why the control cheese of all six series showed lower concentrations of "free" H_2S at 6 months.

Brinkley (1943) also postulated that the same enzyme was involved in breakdown of either serine or cysteine. Wood and Gunsalus (1949), working with partly purified serine deaminase of E. coli, could not confirm this. The finding in L. casei strain 7 of a serine dehydrase which was active in about the same pH range as the cysteine desulfarase could lend some support to Brinkley's postulation of only one enzyme system, since serine and cysteine are very similar in structure. However, many other strains of L. casei showed rather high serine dehydrase activity at pH 5.4, 52° C. but did not produce H_2S in the growth medium. On the other hand, a relationship between the two enzyme systems was indicated when L. casei strain 7, for a short period and for some unknown reason,

failed to produce H_2S in the growth medium and also failed to produce any serine deaminase activity.

Attempts have been made to link flavor development in Cheddar cheese to certain changes in the cheese but so far with little success. Dahlberg and Kosikowsky (1948) linked development of Cheddar cheese flavor to the appearance and increase of tyramine. Tyramine as such was not considered a flavor component but rather taken as an indicator of development of true flavor. This, however, was later disproved by Hupfer et al. (1950) and Dacre (1953a). Barnicoat (1950) demonstrated increases in the number of -SH groups in New Zealand Cheddar cheese with age. He did not place any significance in this, except that presence of more than 35 ppm. of -SH groups usually resulted in off flavors and discoloration of the cheese. The present study indicated a relationship which apparently existed between relative concentration of "free" H_2S and intensity of Cheddar flavor at 6 months. The cheese judged as having the highest intensity of Cheddar flavor also showed the highest relative concentration of "free" H_2S. Perhaps H_2S is a compound which can be used as an indicator of Cheddar flavor development and at the same time occupies a place as a true component of Cheddar cheese flavor, adding both to the aroma and flavor of Cheddar cheese. The importance of H_2S formation in Cheddar cheese was indicated by the isolation from high quality Cheddar cheese of L. casei

types capable of producing H_2S and the apparent lack of these types in lower grade cheese.

Reduced-anaerobic conditions were found to stimulate serine deaminase activity and to be particularly effective for cells which had undergone pasteurization treatment and for cells still in the logarithmic phase of growth. This could indicate oxygen is particularly harmful to the enzymes when the cells are injured or in a stage of growth where the enzymes presumably are very active. The microenvironment of Cheddar cheese is anaerobic and reduced and probably is significant in promoting growth and physiological activity of lactobacilli. The failure of reduced glutathione and adenosine-5-phosphate to stimulate or prolong the serine deaminase activity of L. casei strain 7 was not taken as an indication that these compounds are not required for deamination of serine, as found by Wood and Gunsalus (1949) with E. coli. These investigators worked with partly purified enzymes and similar work remains to be done with L. casei in order to definitely establish which cofactors are required in deamination of serine.

The disappearance of serine during the early months in the cheese to which DL-serine had been added and the appearance of H_2S in all the cheese could be a confirmation of the deamination results found in the laboratory. The added increase in the numbers of lactobacilli in cheese b both of Series I and

III, the cheese with DL-serine added but without addition of L. casei, indicated the ability of lactobacilli to utilize serine as a source of energy. Baker and Nelson (1949) found similar increases of lactobacilli in Cheddar cheese with DL-serine added and also found this resulted in a plus in flavor. This last effect was confirmed in Series I of the present study but not in Series III. The system of cheese ripening is very complex and probably some uncontrolled factor caused the fermentation in Series III to change in an unfavorable direction between the third and sixth months. The beneficial effect addition of DL-serine and phosphatase preparations apparently had on the quality of the cheese inoculated with L. casei could not be related to changes in the population of lactobacilli. Probably changes in the population which might have become evident had the milk not been inoculated or only small inocula used were masked by the rather large inocula.

The cheese with lactobacilli added showed a decrease in numbers of lactobacilli at 3 and particularly at 6 months. Cheese b of Series III also showed a considerable decrease at 6 months. Such decreases in the numbers of lactobacilli after some months of curing have often been reported. Whereas the decrease in the numbers of lactobacilli during the early stages of curing in the cheese with lactobacilli added possibly could be attributed to lack of sufficient amino acids to

support growth of such large numbers, decreases of lactobacilli in regular make Cheddar cheese probably is not caused by that, as amino acids increase in both numbers and concentration as the cheese matures. Teeri and Josselyn (1953) found an excess of glutamic acid repressed growth of L. casei completely and an excess of arginine or histidine caused about 50 per cent repression. As the amounts of the different amino acids increase in Cheddar cheese it becomes apparent that some do not increase as rapidly as others. The reason could be that some amino acids are present in smaller amounts than others in casein or that some are utilized at a faster rate. The sequence of the amino acids in the protein chain and the specificity of the enzymes involved in liberation are other factors which must be considered. It would be difficult to establish the amounts of the different amino acids which would constitute an imbalance in a system such as Cheddar cheese. However, the quantitative studies by Kosikowsky (1950b) showed that free glutamic and basic amino acids were present in Cheddar cheese in amounts considerably higher than the rest of the amino acids at 3 months and beyond, and, in view of the results of Teeri and Josselyn, this could indicate that an imbalance of amino acids could be partly responsible for the decrease in the numbers of lactobacilli encountered in Cheddar cheese after some months of curing.

The results from determination of phosphatase activity at pH 5.4 indicated that any beneficial effect the addition of phosphatase preparation may have had on the quality of the cheese likely took place in the cheese vat. The phosphatase activity determinations on whey and cheese showed that the additional activity of the milk was removed with the whey. Some additional bacterial activity did take place in the cheese with phosphatase preparations added, as demonstrated by the presence of more "free" H_2S at 6 months, but the possibility of the whey globulins and the potato phosphatase preparations contributing something other than increased acid phosphatase activity which could be beneficial to L. casei cannot be overlooked.

Cheese without DL-serine added did not show presence of serine until the third month. As the serine possibly was utilized as fast as it was released, it could not be established if the superior quality of cheese with phosphatase preparations added was due to increased availability of serine during this period. That serine is released during the early stages of Cheddar cheese ripening when the predominant flora normally is made up of S. lactis, is indicated by the work of van der Zant and Nelson (1954). These investigators found that serine was one of the amino acids released by S. lactis, when this organism was grown in fresh skimmilk and presumably the same would happen in Cheddar cheese. The increases in the

numbers of lactobacilli in the Cheddar cheese with DL-serine added could indicate the importance of more available serine in the early stages of ripening of Cheddar cheese made from pasteurized milk. The additional supply presumably would have to come from phosphoserine but, as was demonstrated, the presence of the phosphoric acid group inhibits deamination. Because of the comparatively low phosphatase activity of milk in the acid range and the removal of most of this activity with the whey, it is possible that perhaps phosphatase activity prior to pasteurization has a more important part in removal of ester phosphorus than does the acid phosphatase after the cheese is made. The early results by Graham and Kay (1933) showed that ester phosphorus is released in milk which is left standing at room temperature after milking. Immediate cooling of the milk prevented release of ester phosphorus. These results lend themselves to some interesting speculation.

In recent years Cheddar cheese has been lacking in flavor, as mentioned before. This has in part been blamed on pasteurization and a reduction in the number of bacteria. However, another trend in the cheese industry has been toward producing better and better milk by cleaner handling and by immediate deep cooling of the milk. It is well known that very carefully handled milk is not good for cheesemaking, even for raw milk cheese. This could be due to the low number of bacteria in such milk, but could indeed also be due to insufficient oppor-

tunity for the phosphatases to remove ester phosphorus from casein, thus creating a shortage of serine for energy for lactobacilli. Combined with pasteurization, immediate deep cooling of milk for cheese would leave very little opportunity for release of ester phosphorus. Direct evidence of this hypothesis is not available. However, ripening of the raw milk prior to the beginning of the actual cheesemaking has always been found beneficial, if the milk was not too contaminated with gas producers, although this method is not used in modern factories due to the time consumed. With the introduction of pasteurization, cheesemakers soon discovered heating of the milk to temperatures just below pasteurization resulted in better cheese than heating to the actual pasteurization temperature. One reason could be that the alkaline phosphatase was not completely destroyed and the activity of the acid phosphatases was less affected.

Other indirect evidence is the result obtained by Morris et al. (1951) in treating raw milk for cheese with hydrogen peroxide. Such a procedure resulted in cheese (Swiss, Cheddar and Jack) which ripened faster and had a finer flavor than cheese made from raw and pasteurized milk. The hydrogen peroxide destroyed the anaerobic and coliform bacteria, but was reported not to affect the desirable enzymes found in milk. The authors did not state which enzymes they had in mind, but one group could be the phosphatases. The time and temperatures

employed in the procedure would have given the phosphatases ample opportunity to release ester phosphorus from the casein, and due to the treatment, more ester phosphorus should have been released in the treated milk than in either the raw or pasteurized.

The possible role of the milk phosphatases may be only that of releasing ester phosphorus but could also be that of rupturing the phosphodiester bond reported present in the beta fraction of casein by Perlmann (1954).

The possibility that the differences in the quality of the cheese could have been caused by factors other than those introduced cannot be excluded because the cheesemaking experiments were few and the differences not consistent or large enough to be definite, only indicative. Since it is possible to find rather large variations in the quality of different vats of Cheddar cheese made under identical conditions, a larger number of cheese than was made in this study is usually required before definite effects of variations in the cheesemaking procedure can be established when the differences are small.

SUMMARY AND CONCLUSIONS

1. Lactobacillus casei strain 7 was capable of releasing NH_3 from serine, cysteine and asparagine. Deamination of serine also resulted in increases in pyruvic and lactic acids. Substitution of the hydroxyl hydrogen with a phosphoric acid group resulted in inhibition of deamination of serine.
2. H_2S was released in V-8 juice cultures without added carbohydrate. Both the ability to produce H_2S and the ability to deaminate serine were depressed by addition of glucose to a medium not containing V-8 juice.
3. Temperature and pH studies revealed the possible presence of two serine dehydrases, one with an optimum at pH 7, 52° C. and one with an optimum at pH 8.3, 46° C.
4. Addition of 100 gamma of cysteine and gassing with nitrogen to create anaerobic-reduced conditions in the 2 ml. of test substratum stimulated deamination of DL-serine. Addition of 50 gamma of either adenosine-5-phosphate or reduced glutathione resulted in reduction of deaminase activity.
5. The physiological state of the cells influenced the serine deaminase activity. As the cells grew physiologically older up to the stationary phase the enzyme activity increased when tested under aerobic conditions but

decreased when tested under anaerobic-reduced conditions. However, the cells always showed more activity under anaerobic-reduced deaminase test conditions.

6. Pasteurization of cells decreased the serine deaminase activity very markedly at both pH 5.4, 52° C. and pH 8.3, 46° C. The ability to deaminate DL-serine was in part recovered under anaerobic-reduced deaminase test conditions. The extent of inactivation by pasteurization and recovery by anaerobic-reduced test conditions seemed to be determined at least in part by the physiological state of the cells.
7. Serine deaminase activity of cells, when growth was stopped at pH 5.0, was most stable when the cultures were stored at pH 8.3 under aerobic conditions. At pH 4.2 enzyme activity decreased very markedly.
8. Lactobacillus casei types capable of producing H_2S and of deaminating DL-serine were isolated from high grade Cheddar cheese. Types which could only deaminate DL-serine were found in lower grade cheese.
9. A total of six series of Cheddar cheese was made. Two were to study the effect of addition of DL-serine to curd made from pasteurized milk with and without Lactobacillus casei added. The other four series were made to study the effect of addition of whey globulins containing the acid milk phosphatase and potato phos-

phatase preparations to pasteurized milk inoculated with Lactobacillus casei. The cheese were examined at 2 days and 1, 3 and 6 months for phosphatase activity at pH 5.4, numbers of lactobacilli, pH, protein breakdown, -SH groups and "free" H_2S, free amino acids and quality.

10. The results of the cheesemaking experiments as a whole indicated the possible role of serine as a source of energy for lactobacilli in Cheddar cheese. They also indicated a connection between utilization of serine and production of H_2S which is reflected in the quality of the cheese. Cheese in which serine apparently was utilized early contained more "free" H_2S and was of a higher quality than cheese in which serine apparently continued to be utilized; such cheese showed less "free" H_2S and was of a lower quality. Cheese made from milk with phosphatase preparations added were of superior quality at 6 months, but the role of the acid phosphatase was not definitely established. The possibility of the milk phosphatases having an important part in ripening of Cheddar cheese (and other types of cheese) prior to pasteurization was discussed.

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