THE INFLUENCE OF NUTRITIONAL FACTORS AND OF MUTATIONS ON THE RATES OF ACID PRODUCTION BY LACTIC STREPTOCOCCI

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CHAPTER I

INTRODUCTION

Lactic cultures are used extensively in the dairy industry for the manufacture of cheese and the production of buttermilk and other fermented milks. The lactic streptococci, which predominate in these cultures, having the enzyme B-galactosidase, are able to hydrolize part of the milk sugar (lactose) into glucose and galactose and through glycolysis produce lactic acid. The acid produced will then coagulate casein and as a result curd is formed. Since rapid acid production has a marked effect in reducing the time required for cheese manufacturing, the development of practical means by which both the rate and total acid production can be increased would be an important contribution to the dairy industry.

Although milk is used almost universally for routine propagation of lactic cultures, it is not an ideal medium because of variations in composition from day to day. Understanding the metabolic pathways and determining the environmental (nutritional) factors governing the enzymatic reactions of the cell will be helpful in perhaps partial or full achievement of our goals. Since it is difficult or even impossible to remove single nutritional components from a complex medium such as milk, an exact determination of the growth factors requires the use of a completely chemically defined medium. Such a synthetic medium might be used in place of milk to avoid the hazard from occasional presence of

antibacterial substances and the high calcium content of milk which is favorable to bacteriophage development.

Since metal ions play important roles in the biochemical phenomena due to their association with both large (protein and nucleic acid) and small (i.e. adenosine triphosphate) organic molecules, and the fact that there is general agreement among workers concerning requirements for vitamins for lactic streptococci, there is need for information as to the requirement of these organisms for certain metal ions. Furthermore, since the addition of certain supplementary materials, notably pancreas extract, has been shown to stimulate growth of these cultures, it seems logical that the addition of intermediary metabolites might have a similar or perhaps more pronounced stimulatory effect.

Due to the fact that Escherichia coli constitutive mutants capable of making 25% of total protein as B-galactosidase have been isolated, and that this enzyme is an inducible enzyme in the lactic streptococci, it was thought that perhaps the isolation of lactic streptococci constitutive mutants would have valuable practical application to the dairy industry, due to the increase of acid production by these streptococci.

The objectives of this research on lactic streptococci herein reported were: (a) to determine the mineral requirement for growth, (b) to develop a medium for the propagation of these cultures, (c) to determine the influence of certain additives on rates of acid production, and (d) to isolate B-galactosidase constitutive mutants capable of rapid acid production.

CHAPTER II

REVIEW OF LITERATURE

Two groups of proteins which associate with metals can be differentiated. In one group, the metalloproteins, a given metal is an integral part of the protein structure and cannot be removed without destroying such structure and stoichiometric amounts of the metal follow the protein through the ultimate steps of purification (17,25,67,68). The iron proteins, the copper proteins, and the zinc proteins are examples. Studying those enzymes which depend on metal ions for their activity appears to offer an approach in understanding the nature of enzyme catalysis in chemical and physical terms (25).

Vitamin B_{12} (cyanocobalamin) is potentially related to this group of substances possibly as a prosthetic group (57). This vitamin is a cobalt coordination complex containing one replaceable cyano group which is tightly and coordinately bound to the cobalt atom (8).

In the second group, the metal-protein complexes, the metal ion combines reversibly with the protein molecule in such a way as to stabilize a particular conformation of the protein moiety. The effect of calcium on casein and on the hydrolysis of proteins by trypsin may be examples of such interactions. There are proteins such as conalbumin of egg white and transferrin (siderophilin) of blood plasma which bind certain metal ions tightly but reversibly; such complexes may be involved in the transport and metabolism of iron and other metals (17).

Metal ions typically interact with two or more ligands: two ligands in a linear array, four ligands with the metal ion in the center of either a tetrahedron (zinc) or plane (cupric), or six ligands which are disposed about a central metal ion at the corners of an octahedron (33). Based on the logarithms of association constants for the formation of 1:1 metal-ligand complexes, the stability sequence of several divalent metal with a given ligand is, for almost every ligand (2,3,33),

$$Ca < Mg < Mn < Fe < Co < Ni < Cu > Zn$$

This order is broken when coordination numbers exceed 4, partly because cupric and zinc ions tend to be only four-coordinate with ligands other than water and also since sulfhydryl ligands tends to promote Zn.

Mg^{++}, Ca^{++} and Mn^{++} are likely to be bound to charged oxygen ligands. An exception is the coordination of Mg with nitrogen donors in chlorophyll molecules; this seems to be influenced by the abundance and small size of Mg. Fe^{++}, Co^{++} and Ni^{++} tend to coordinate with oxyanion-nitrogen ligands, while Cu^{++} and Zn^{++} prefer sulfur and nitrogen ligands (33,72,73).

The same data (2,3,33) showed that the formation constants for ligands with more than one binding site were much higher than for those with only one. Furthermore, the sum of two association constants for a monodentate ligand was less than the corresponding single constant for a bidentate ligand, i.e., the formation constant for ethylenediamine was greater than the sum of two constants for ammonia. Similarly, the constant for glycine was greater than the sum of constants for both ammonia and acetic acid, and thus glycine bound metal ions better than a mixture of acetic acid and ammonia.

A single ligand with two or more donor groups is capable of forming

a chelate compound with a metal ion (17). Thus, all amino acids exhibit chelate formation. With the exception of histidine and cysteine, which form a variety of chelate structure, most amino acid have similar formation constant (2,3,33,72,73). These two amino acids have larger formation constants and thus bind metal ions better than the rest of the amino acids and are probably more involved in forming metal-protein complexes.

Since nucleic acids are negatively charged molecules, they must be accompanied by a number of cations so that electrical neutrality may be attained by such structures. Smith and Alberty (59), using various adenosine phosphates found that Li^+ complexes with the former more than any other monovalent cation, followed by Na^+ and K^+. Such metals serve only to neutralize the negative charges on the phosphate residues of nucleic acids electrostatically. This then allows maximum compact conformation of the macromolecules (30). The alkaline earths, certain transition metals such as Co^{++}, Mn^{++}, Ni^{++}, and Zn^{++} on the other hand are bound stoichiometrically, perhaps to the phosphate residues. Within their stoichiometric range these divalent metals are much more effective than the monovalent in bringing about the compact conformation of the nucleic acids (30).

Zubay (76) obtained a three-stranded polynucleotide while working with synthetic polymers. He then proposed a theory for nucleic acid structure based on the formation of complexes between magnesium ions and adenine bases. Other metals such as Hg^{++}, Cu^{++}, Fe^{++} and probably Pb^+ react with some of the nucleic acid bases as well as the phosphate residues (30).

Most, if not all, of the enzymatic reactions involving adenosine

triphosphate (ATP) require the presence of a divalent metal ion in stoichiometric amounts. This requirement is usually met by the participation of magnesium ions (6).

Cytochrome c and cytochrome a were obtained in pure crystalline form, and it was found that they contained iron and copper, respectively. These cytochromes, which are components of the respiratory chain, play important roles in respiration. Although there has been some controversy as to whether mitochondria exist in bacteria, it is now clear that the respiratory chain enzymes are located in the protoplast membrane (26).

Sulfur is found in coenzyme-A (61) and in the amino acids methionine, cysteine, etc. Cysteine contributes an -SH group near or at the active site of a number of enzymes and provides the reactive group in coenzyme-A and glutathione (50). Sulfur and selenium both belong to group VI of the periodic table. Simple selenium anologues of sulfur compounds of biological importance have been identified as follows: seleno-methionine, seleno-methylcysteine, seleno-cysteine, seleno-coenzyme-A, seleno-cystathionine, and seleno-taurine. Inorganic selenite seems to be well incorporated into these organic sulfur compounds, possibly by reduction to H_2Se and then -SH:SeH exchange. Selenium also exists in other organic forms which appear not to be sulfur anologues (14,61).

Glycolysis in Lactobacillus arabinosus was stimulated by K^+ or Rb^+, but inhibited by Na^+, Cs^+, or NH_4^+. This inhibition was overcome by addition of K^+ or Rb^+, and its degree was proportional to the ratio of inhibiting to stimulating ions. In the same way Zn^{++} inhibited glycolysis and its effect was overcome by Mn^{++}. Furthermore, Mg^{++} and Ca^{++} did not oppose the Zn^{++} inhibition (65).

Pamir (52) selected certain lactic acid bacteria, on the basis of

their inability to thrive in a medium deprived of metallic ions, and propagated them in media containing varying concentrations of the ions to test the growth-promoting and inhibitory effects produced by different metals. Mn, Fe, and Mg stimulated the growth of Lactobacillus casei, while Cu and Zn were toxic at concentrations of 100 µg./5 ml. Mg at high concentration was the only metal which stimulated the growth of L. bulgaricus. Combinations of different metals often gave opposite effects. EDTA could prevent the toxic effects of Cu and Zn on L. casei, but with L. bulgaricus only Zn toxicity was removed.

Nowakowska-Waszczuk (46,47) stated that Mg appeared to be essential for growth of L. lactis and L. delbrueckii and that Ca, Fe, and Cu were unable to substitute for Mg in these cultures. The growth of L. delbrueckii was inhibited even at low concentrations of Mn, but in cultures of L. lactis, Mn could partially substitute for Mg.

It was found that L. acidophilus could not metabolize EDTA chelates. Mn and Mg appeared to be the only cations required by this organism (56).

Experiments to determine the effects of sodium chloride on lactic streptococci have generally been concerned with its influence on growth or acid production by these organisms. The effect on growth appears to be relatively clear, when widely different salt concentrations are considered and has been used by Sherman (58) as one criterion for identification of Streptococcus lactis and Streptococcus cremoris. The former will grow in the presence of 4% salt, whereas the latter will not. The influence of salt on acid production is less clear since results were obtained under widely different conditions. McDowall and Whelan (36) cited work by Orla-Jensen, who found that most lactic acid bacteria were not inhibited by 2.5% salt in peptone broth with 2% added dextrose. He

noted considerable inhibition with 5.5% salt and complete inhibition with 10.0% salt. Sokol' Skaya (62) observed that acid production by S. lactis propagated in skim milk fortified with different level of salt was stimulated with 0.5% and depressed by 2% NaCl. The tolerance of the organism was improved with increased buffer capacity and nutritive value of the growth medium.

The results of tests conducted by McDowall and Whelan (36) on pure and mixed lactic cultures, using whole milk, showed variation from experiment to experiment, but there was always a stimulation of acid production by 1.0% and frequently by 2% salt, whereas 3.0% was generally inhibitory. Irvine and Price (22) observed that the acidity of six commercial cultures growing in reconstituted nonfat dry milk was not reduced by the presence of up to 1.0% salt; slight reduction occurred with 1.5% salt; substantial reduction took place when 2.5% or more salt was present, and some cultures were stimulated slightly by the presence of 0.5% NaCl. More recently, Marth and Hussong (32) utilized skim milk fortified with 1.0% NFDMS and 1.5% fat for their studies, and observed that at 22.2°C and with 1.0% inoculum, the rate of acid production was: (1) not affected by 0.25%; (2) reduced slightly by 0.5%; and (3) reduced appreciably by 0.75%. The acid production was reduced when 1.0% or more NaCl was present with 5.0% inoculum at 22.2°C and/or 1.0% inoculum at 30°C. No appreciable difference in acid production rate was noted in milks with 0.75% or less NaCl, when a 5.0% inoculum and 30°C incubation were used, whereas 1.0% to 1.5% salt was accompanied by a slight reduction.

Whiteside-Carlson and Rosano (70) found that manganese and phosphate ions were essential for Leuconostoc dextranicum growing in various carbohydrate media, and incubated at 25°C for 96 hours.

Galesloot and Hassing (15) examined aroma bacteria in butter starters over a period of one year, and found that the development of S. diacetilactis was not affected by season, whereas, that of Betacoccus cremoris was considerably lower in the spring than in the autumn. This was thought to be related to a stage of lactation, and the growth of this organism in the spring milk was stimulated by the addition of Mn to the milk.

Since a 24 hour starter was negative to the creatine test if Mn was added to the milk and positive if none was added, even when the only aroma-forming bacteria present were S. diacetilactis, it was concluded that Mn affects the ability of bacteria to reduce the aroma compounds. The addition of Mn to milk with fairly high and fairly low Mn contents indicated that it was the available Mn, rather than the total Mn content which affected the reduction (51).

The addition of vegetable extracts to a growth-sustaining medium for subsequent enhancement of growth of many bacteria has been used. Tomato juice was separated by chemical and physical methods into various active fractions, as measured by growth response and acid production by numerous lactic acid bacteria (63). The stimulatory component was inorganic, and of the various cations tested, Mn was the only element producing biological activity comparable to that of the original extract. Of 71 strains tested, 63 showed a definite requirement for Mn or tomato juice. Stamer, et. al. (63) cited work by Zlataroff and Kaltschewa who noted that the addition of manganese salts to a milk medium produced greater acidity with a S. lactis culture.

It was found that 2 ppm of manganese added to culture milk stimulated the growth of Leuconostoc. Furthermore, mixed cultures grown in

milk with 2 ppm manganese showed a higher activity rating than those grown in milk without the added manganese. With pure cultures of S. lactis types no such stimulation was apparent (48).

In a recent experiment, using a synthetic medium, two strains of S. lactis (ML_3 and C_{10}) and one strain of S. cremoris (HP), Reiter and Oram (55), found that Fe is required by cultures HP and C_{10} in a concentration of .01 and .004 ppm. respectively. Culture ML_3 gave inconsistent results and often growth occurred in the absence of added iron. The iron requirement by these microorganisms can be satisfied alternatively by vanadium, while molybdenum, cobalt, zinc and copper failed to replace iron or vanadium. Furthermore, they stated the findings of Reiter (unpublished) that magnesium and potassium were essential for the growth of these lactic streptococci and that these organisms failed to grow in milk deionized with IRC 50 resin (Sodium form). The same findings were obtained in preliminary experiments using a synthetic medium.

More recently, Olson and Qutub (49) found that sea salt and kelp extract (50 gm dried kelp/150 ml H_2O) stimulated acid production by lactic streptococci, when the skim milk was fortified with 0.4% of the sale and/or 0.4 ml/10 ml of the kelp extract. Preliminary results for screening 12 mineral elements indicated that iron, magnesium, molybdenum and selenium were stimulatory to acid production by these organisms; cobalt and zinc appeared to be slightly stimulatory, while boron, lead and lithium had no effect or were slightly inhibitory. Copper, iodine and mercury were inhibitory, with pronounced inhibition occurring at levels of 4 and 8 ppm. On the basis of these findings, iron, magnesium, molybdenum and selenium were set up in a 3^4 factorial a-rangement. The analysis of variance showed that iron, magnesium and selenium significantly ($P < 1\%$)

increased the rate of acid production by lactic streptococci in milk, while molybdenum had little or no effect. They concluded that the addition of 2 ppm of iron, 4 ppm of magnesium, and 0.5 ppm of selenium to milk will significantly improve the rate of acid production by these streptococci.

Niven (40) was the first to study the nutritional requirements of the lactic streptococci in a completely synthetic medium. He reported that the omission of leucine, isoleucine, methionine, valine, and arginine individually from a complete amino acid mixture exerted a pronounced effect on growth of a single strain of S. lactis, but the simplest combination that allowed growth comparable to that in the complete amino acid mixture consisted of 14 amino acids. Glutamine and asparagine were essential for the initiation of growth in all 21 S. lactis strains tested using hydrolyzed casein. However, Pollack and Lindner (53) found that aspartic acid and asparagine were not required by any strain of S. lactis which they investigated and that glutamic acid could replace the essential amide if a high concentration was employed. Anderson and Elliker (4), in a similar nutritional investigation of 35 strains of S. lactis and S. cremoris, showed that all strains required glutamine, leucine, isoleucine, methionine, valine, histidine, proline, and all but one required arginine.

Early studies concerning proteolysis by S. lactis in milk were reviewed by Hammer and Babel (18). Collins and Nelson (11) reported that S. lactis grown in skim milk caused a considerable increase in the trichloroacetic acid-soluble nitrogen during the first 24 hours, with gradual but smaller increase throughout the 15-day test period. Morgan and Nelson (39) reported marked increases of ten amino acids in tungstic

and lactic acid filtrates of milk after incubation with S. lactis for 15 days at 21°C. Vanderzant and Nelson (69) found that S. lactis caused a rapid increase in both soluble nitrogen and tyrosine and tryptophan during the first 24 hours, followed by a smaller but gradual increase during the rest of the incubation period (72 - 90 hrs.). Considerably more soluble nitrogen and tyrosine and tryptophan were produced when the reaction was controlled at pH 6.0 - 7.5 than without controlled pH. Furthermore, the cell-free culture medium did not show proteolytic activity.

The ability of lactic streptococci to utilize unhydrolyzed sodium caseinate as the nitrogen source in an otherwise chemically defined medium seems to be a strain rather than a species characteristic (35). Growth of this sort adds up to the previous findings (5,11,39,69) that these organisms produce an extracellular proteinase. Growth of lactic streptococci in a medium with unhydrolyzed sodium caseinate did not appear to be related to their minimal amino acid requirements. In fact, they did not necessarily require fewer amino acids than those that failed to utilize caseinate. Some strains that were unable to use sodium caseinate required fewer amino acids for growth in the presence of the protein than in its absence. The sparing effect observed varied with the strain. However, leucine, isoleucine, methionine, and valine appeared to be essential for this group of organisms in the presence or absence of the caseinate (20).

In recent experiments using the single omission technique, Reiter and Oram (54) found that glutamic acid, valine, methionine, histidine, leucine and isoleucine were essential for the growth of all 26 lactic streptococci tested. Aspartic acid, citrulline and ornithine were not

required by any. All 18 strains of S. cremoris required proline and phenylalanine; most of them also required or were stimulated by tyrosine, serine, lysine and alanine and a few by threonine and tryptophan. Only one required glycine. Some strains of S. lactis and S. diacetilactis required arginine and phenylalanine.

A zone isolated by paper chromatography from the spent broth of a mixed strain lactic streptococcus culture was found to be inhibitory to the culture. The eluted material from the inhibitory zone was identified as leucine by thin-layer-chromatography. Exogenous addition of D- and L-leucine revealed that D- leucine and not L-leucine is the inhibitory factor (16).

Snell and Mitchell (60) found that adenine and thymine appeared to be essential for growth of S. lactis. Stokes (64) observed that thymine or thymidine could replace folic acid in the growth of S. lactis R (S. faecalis), but that adenine was necessary for active growth with either thymine or folic acid. Guanine or xanthine could substitute for adenine. Niven (40) found that the purine and pyrimidine bases function as growth stimulants. Any one could be omitted without serious difficulty, but growth was retarded when all were omitted.

Dahiya and Speck (13) reported that the addition of inosine or adenosine increased the rate of acid production by lactic streptococci in steamed milk more than any other purine or purine riboside tested. The pyrimidines showed no effect. However, Kristoffersen (24) using lots of pasteurized milk fortified with either xanthine, hypoxanthine or uric acid, found that xanthine retarded the rate of acid production by lactic streptococci in one trial, while hypoxanthine almost completely prevented acid development in another trial using different lots of

milk. He then concluded that the effectiveness of these purines varies with milk supply. The addition of the purines to raw milk and storage for 5 hrs. at 37 C (with or without pasteurization afterward) nullified their inhibitory effect. From these and the fact that uric acid did not show any retarding effect, he concluded that perhaps xanthine oxidase activity resulting from the added substrates was the cause of the observed decrease in the acid production by these streptococci. The above findings that the inhibitory effect of xanthine and hypoxanthine varied with milk supply may be explained by the demonstration of Hwang, et. al. (21) that milk contains activators and inhibitors for the enzyme xanthine oxidase. Heating the milk to 91.4 C for 4.2 sec. inactivated the enzyme, but did not destroy the activators nor the inhibitors.

The polynuclear aromatic planar cationic (basic) dyes of which the acridines (proflavine, acridine orange, etc.) have long been used as biological stains due to their selective binding to and rendering visible DNA or RNA by virtue of their absorption or fluorescence. Such dyes are now being used as highly selective mutagens. Brenner, et. al. (7) state that the mutagenic functions of these dyes is due to the insertion or the deletion of a base-pair. This is supported by the findings of Lerman (27,28) that the acridines are bound to DNA by sliding between adjacent nucleotide-pair layer (intercalation) thus forcing them 6.8 $\overset{o}{A}$ apart, rather than 3.4 $\overset{o}{A}$. If this occasionally happened between the bases on one strand of the DNA, but not the other, during replication, it might easily lead to the addition or subtraction of a base. Furthermore, the sequence of the bases for the genetic code is read from a fixed starting point, and there are no special "commas" to show how to select the right triplets. If the starting point is displaced by one

base, then the reading into triplets is displaced, and thus becomes incorrect (12).

Mandell and Greenberg (31), the discoverers of N-methyl-N-nitro-N-nitrosoguanidine (NTG) as a mutagenic agent, had eliminated the possibilities of nitrous acid (acid decompositions) or diazomethane (alkaline decompositions) production to account for NTG mutagenicity. Rather, they concluded that the NTG structure is the mutagenic agent. Adelberg, et al. (1), reported that NTG acts as a powerful mutagen and induces at least one mutation per cell under conditions in which over 50% of the cells survive. Significant killing occurred only if the cells were allowed to grow and metabolize in the presence of the mutagen. Since the rate of induced mutation was the same whether the cells were washed and treated in buffer or were treated in a medium permitting active growth, it seemed unlikely that DNA replication must occur in the presence of NTG in order for mutations to be induced. Rather, NTG may react with DNA and alter it in such a way as to promote template error during subsequent replication.

The synthesis of enzymes in bacteria follows a double genetic control. On one hand are the so-called structural genes which determine the molecular organization of the proteins, while the regulator and operator genes, control the rate of protein synthesis through the intermediacy of protein components or repressors. The repressors can be either inactivated (induction) or activated (repression) by certain specific metabolites. This system of regulation operate directly at the level of enzyme synthesis by the genes of a short-lived intermediate, or messenger RNA, which becomes associated with the ribosomes where protein synthesis takes place (23).

In these (23) and other studies (19) constitutive mutants have been isolated for several inducible systems. These mutations are located in either the regulator or the operator genes. Such mutants are capable of synthesizing a larger amount of the enzyme in question than normally made. Of particular interest are the so-called hyper constitutive mutants of E. coli strains which are capable of making as much as 25% of the total protein as B-galactosidase. These mutants had been isolated by Horiuchi, et al. (19) using a continuous flow device, the Chemostat (41) in which the bacterial population was maintained over an indefinite period of time by maintaining the concentration of one of the growth factors, called the controlling growth factor, at a low fixed value. These workers (19) demonstrated that these changes are genetically controlled, since they can be transferred by genetic recombination. Recently B-galactosidase non-inducible mutants of E. coli K12 have been isolated which have lost the capacity to be induced by B-galactosides. The location of these mutations are in the regulator gene of the Lac region (74).

Novick and Szilard (42) observed that spontaneous mutations of E. coli (B strain) occurred in the Chemostat, and that if back mutations can be neglected, the mutant population in the Chemostat will increase linearly with time, over periods of time short enough to disregard "evolutionary" changes. Therefore, plotting the number of mutants against time will give a straight line whose slope is proportional to the mutation rate.

In similar experiments Novick and Szilard (43) found that the purine derivatives, caffeine, theophylline, paraxanthine, and theobromine added to the nutrients in the Chemostat have a marked effect on the mu-

tation rate, while 2-6 diamino purine did not. Adenine also induced mutation rate to a lesser extent. They also found that there is a similar dependence of the mutation rate on the choice of the controlling growth factor for the chemically induced mutation as for the spontaneous mutation. On the other hand none of the pyrimidines or their derivatives (uracil, thymine, 6-methyluracil, 5-aminouracil or 5-bromouracil) appeared to be mutagenic in a concentration of 150 mg per liter. From these experiments, they concluded that there is no correlation between the mutagenicity of a compound and its presence as a constituent of nucleic acid. It was found that certain ribosides such as adenosine, guanosine and inosine counteract strongly the mutagenic action of caffeine and other mutagens (44).

Wilkowske and Fouts (71) reported a continuous growth of lactic cultures in a system resembling that of the Chemostat, with pH as the governing factor, for the purpose of continuous production of fermented products. The system operated for 12 weeks, but not without contaminations and undesirable fermentations, followed by a successful trial for a period of 3 weeks.

Novikova (45) found that 3 out of 4 S. lactis cultures studied, were able to hydrolyze lactose into glucose and galactose and therefore have lactase. Cetti, et. al. (9) reported that in S. lactis the synthesis of B-galactosidase was induced by lactose. Only one out of 50 strains produced the enzyme with sufficient stability to permit purification (37).

Methyl-B-D-thiogalactopyranoside, isopropyl-B-D-thiogalactopyranoside, and galactose induced the production of B-galactosidase, but less effectivily than lactose. Melibiose, maltose, and calcium lactobionate

were poor inducers of enzyme synthesis (9). From radioactive lactose and substrate uptake studies, the same workers (10) concluded that specific permeases and metabolic enzymes were induced by lactose and maltose in S. lactis.

Vakil and Shahani (66) indicated that S. lactis UN posesses lactase (B-galactosidase) and lactose dehydrogenase activities. Furthermore they stated that this strain is capable of utilizing lactose by possibly two pathways: hydrolytic cleavage by lactase or B-galactosidase to yield glucose and galactose; and by oxidation via lactose dehydrogenase to lactobionate, before its enzymatic cleavage to gluconate and galactose.

Woodard et al. (75) reported that the addition of B-galactosidase to milk increased the rate of acid production by lactic streptococci. Furthermore, single strain of S. lactis showed preference for glucose in growth experiments using broth containing glucose, galactose, or lactose.

CHAPTER III

EXPERIMENTAL METHODS

Source and Propagation of Lactic Streptococci

The pure cultures of S. lactis type organisms used in the work herein reported were obtained from the stock of cultures carried in the Dairy Science Laboratory at Oklahoma State University. These cultures represented both S. lactis and S. cremoris that had been isolated from commercial lactic cultures and were selected, among other characteristics, for their abilities to produce acid rapidly in milk. The cultures were maintained in an active condition by propagating in sterile litmus milk contained in test tubes, using about 1% inoculation and incubating at 22 C for 16 hours. The cultures were propagated at least twice a week and only freshly incubated cultures were used in the experimental work. When needed the cultures were propagated 2-3 times successively in a broth medium.

Four-hour Activity Test

The four-hour activity test is considered a good indication of the expected performance of a culture in cheesemaking because the milk used, amount of inoculation and temperature of incubation are the same as used for the short set method of cottage cheese. Briefly, the method involved the placing of measured 9 ml quantities of pasteurized (73C-16 sec) milk into sterile test tubes (20 x 150 mm), inoculating with

0.5 ml of fresh culture, tempering to 32 C and incubating for exactly four hours. The cultures were then titrated with N/10 NaOH, using one drop of 10% phenolphthalein as the indicator. The results are expressed as the ml of the alkali required to titrate the entire contents of each tube. When large numbers of tubes were used in a trial, the tubes were placed in ice water during inoculation and after the incubation period to inhibit growth and acid production.

Preparation of Stock Solutions

Vitamin Mixture

A vitamin mixture was made by dissolving 0.1 gm of each of riboflavin, calcium pantothenate, nicotinic acid and pyridoxine; 10 mg thiamine HCl and 100 gammas biotin in glass distilled water made up to a volume of 100 ml. The mixture was then sterilized by filtration through a Seitz filter, diluted 1 : 10 with sterile glass distilled water and stored in the refrigerator until needed.

Amino Acid Solutions

One percent solutions of each of 20 amino acids in glass distilled water were prepared and sterilized by filtration through a Switz filter.

Nucleoside and Nucleotide Solutions

One tenth gram of each of adenosine, guanosine, uridine and cytidylic acid were added separately to 99.9 ml glass distilled water and sterilized by filtration through a Seitz filter.

Mineral Solutions

A mineral mixture was prepared by dissolving 12 mg $MnCl_2 \cdot 4 H_2O$, 40 mg $FeSO_4 \cdot 7 H_2O$, 0.8 gm $MgSO_4 \cdot 7 H_2O$ and 2 gm NaCl in glass distilled water and making up to a volume of 10 ml. Also, 10 ml aqueous solutions of each mineral compound were prepared.

Basal Media for Lactic Streptococci

Medium-A	%	Medium-B	%
Glucose	0.5	Sugar (variable)	variable
Hydrolyzed casein	0.5	Hydrolyzed casein	0.5
Adenine	0.001	Adenosine	0.001
Uracil	0.001	Uridine	0.001
Vitamin mixture	1.0	Guanosine	0.001
Mineral mixture	1.0	Cytidylic acid	0.001
$Na_2HPO_4 \cdot 7 H_2O$	0.32	Vitamin mixture	1.0
KH_2PO_4	0.08	Mineral mixture	1.0
		$Na_2HPO_4 \cdot 7 H_2O$	0.32
		KH_2PO_4	0.08

These media were used as broth or as agar media with 1.5% agar in the latter.

Measurement of Extent of Growth

Growth was measured by one of the following methods:

Spectrophotometric Method

The medium was dispensed into test tubes, sterilized, cooled, inoculated with the organism and, after incubation, the amount of growth was determined by turbidity measurement with either a Coleman model 6D spectrophotometer at 540 nm or a Klett-Summerson colorimeter at a wave length of 420 nm.

Auxanographic Method

Agar medium was prepared, sterilized and poured into sterile plates, with about 10 ml per plate. After solidifying and allowing to partially dry overnight to eliminate excess moisture, the plates were marked on the bottom with a wax pencil. The test culture, grown in milk or broth, were diluted about 1:2000 by transferring a small loopful (about .005 ml) into 9 ml of sterile distilled water. One small loopful (about .005 ml) of the diluted culture was then inoculated onto the surface of the agar in the designated portion of the plates and the rate and extent of growth approximated by visual observation after incubation, usually at room temperature (24-28 C).

Relative amount of growth:

-	=	none
+	=	very slight
++	=	fair
+++	=	good
++++	=	very good

Selection of Mutants of Lactic Streptococci

Selection of lactic streptococcus mutants was accomplished by using a chemostat and by treatment with a mutagen agent.

Selection of Mutants by the Use of a Chemostat

The chemostat is a device designed for continuous bacterial growth.

It automatically renews the nutrient medium in a culture vessel suitable for growth of microorganisms by the addition of fresh nutrient to the growing cell populations and concurrently removes a fraction of the bacterial suspension. The contents of the culture vessel are sufficiently stirred so that the entering nutrient medium is quickly and uniformly dispersed throughout the vessel. Sterile nutrient medium with a limiting amount of lactose (0.03%) enters the culture vessel from a reservoir at a constant rate of flow and the liquid in the vessel is maintained at a constant level by means of an overflow tube so that the flow of effluent of bacterial suspension is equal to the rate of inflow.

To check for B-galactosidase constitutive mutants samples of effluent from the chemostat were taken at intervals and grown in the same medium as used in the chemostat but with liberal amount of maltose (or glucose) substituted for the lactose. After incubation the organisms in the medium were treated with toluene and assayed for the presence of B-galactosidase as described below. Samples of effluent were also propagated in sterile litmus milk and evaluated by the four-hour activity test.

Mutagenesis by NTG

Fresh solutions of N-methyl-N-nitro-N-nitrosoguanidine (NTG) (Aldrich Chemical Corp.) were made for each trial by dissolving 10 mg. NTG in 10 ml sterile 0.05 M sodium acetate buffer at pH 6.5. An active S. lactis type culture growing in broth medium was inoculated (5%) to another 9 ml portion of the broth and incubated until the cells were in logrithmic phase. The cells were then harvested by centrifugation, washed twice with buffer solution and resuspended in the 9 ml of sodium acetate buffer. To 4.5 ml cell suspension 0.5 ml NTG was added

to give a final concentration of the latter of 100 µg per ml. The tube was incubated with agitation at 37 C for 30 minutes and the NTG then removed by centrifugation and the cells were washed and resuspended as before. The resuspended cells were diluted and placed in broth containing maltose or other sugars. After growth the broth tubes were assayed for the enzyme. In one trial tubes of sterile litmus milk were inoculated directly from the diluted mutagenized cell suspension, while in a later trial the diluted cells were streaked over agar plates containing lactose, incubated and colonies picked into litmus milk. The cultures obtained from direct inoculation and from picked colonies were evaluated by the four-hour activity test.

Assay for B-Galactosidase

The rate of hydrolysis of the chromogenic substrate O-nitrophenyl-B-D-galactopyranoside (ONPG) (Sigma Chemical Corp.) by toluenized samples of bacteria was used as a measure of the amount of B-galactosidase in that sample. Solutions of 0.002 M ONPG were prepared in sodium phosphate buffer (0.1 M) at pH 7.0. A four ml sample of bacterial suspension was treated with 0.2 ml of toluene-acetone (1:9) solution and incubated for 5 minutes at room temperature with vigorous agitation. This was done to destroy the permeability barrier of the cells and to prevent induction during performance of the test. A 1.0 ml volume of toluenized cells was incubated with 2.0 ml of ONPG solution at 37 C. Color development was stopped at an appropriate time by adding 5.0 ml of 0.5 M sodium carbonate to the reaction mixture. The samples were then read in a Klett colorimeter at 420 nm. The assay readings were then expressed in terms of the amount of enzyme per unit of turbidity

as follows $\frac{\text{assay reading}}{\text{turbidity reading}}$ = Enzyme per unit of turbidity.

CHAPTER IV

RESULTS AND DISCUSSION

Nitrogen Sources for Lactic Streptococci

In order to determine the mineral requirements for lactic streptococci it was deemed necessary to first develop a simple medium which would be adequate for growth of these organisms. It was thought that perhaps ammonium sulfate and urea fortified with certain amino acids might be satisfactory as nitrogen sources for growth of these rather fastidious organisms. Accordingly, trials were conducted to determine the minimal requirements of these materials.

Ammonium Sulfate, Urea and Selected Amino Acids

It had been reported (4, 40, 54) that a relatively few amino acids were required for growth of lactic streptococci but it was not shown that a medium containing only these amino acids as sources of nitrogen would support growth of these organisms. A trial was conducted to determine the influence of adding singly and in combinations asparagine and glutamine and five reportedly essential amino acids. A basal agar medium was prepared for this trial. The medium was composed of 1.5% agar, 0.5% glucose, 0.001% adenine, 0.001% uracil, 0.1% ammonium sulfate, 0.1% urea and adequate minerals and vitamins provided by adding 1.0% of mixtures of these materials (see methods). After sterilization 0.01% and 0.02% of asparagine and glutamine, singly and in combination,

and a mixture of five amino acids were added to portions of the agar medium. The auxanographic method was used to evaluate the influence of the various additives on six lactic streptococcus cultures. The results are shown in Table I.

Two of the six cultures grew well on the medium with no additives, while the remaining four cultures failed to grow. It appeared that asparagine as the sole additive was inhibitory but this inhibition was nullified by the addition of glutamine. The results also show that good growth response was obtained with one culture (H-15) when a combination of 0.02% each of seven amino acids (arginine, asparagine, glutamine, isoleucine, leucine, methionine and valine) was added, while the remaining three cultures which failed to grow in the medium with no additives also failed to grow with the seven amino acids added.

These results indicate that additional sources of nitrogen are necessary for the growth of certain strains of lactic streptococci.

Amino Acids

Since the previous trial indicated that a medium containing seven "essential" amino acids was inadequate for certain strains of lactic streptococci, an experiment was conducted in which the 20 amino acids occurring in casein were used as sources of nitrogen. The same basal medium was used as in the previous trial except that the ammonium sulfate, urea and agar were omitted. The amino acids were added in concentrations ranging from 0.007 to 0.02% with most of them added at the 0.01% level. Ten pure cultures of lactic streptococci were used to inoculate the tubes into which the medium had been dispensed. The spectrophotometer was used for measuring the extent of growth at inter-

TABLE I

GROWTH OF LACTIC STREPTOCOCCI ON A MEDIUM CONTAINING AMMONIUM SULFATE AND UREA AND FORTIFIED WITH AMINO ACIDS

Amino Acid Added	Relative Amount of Growth (100 hrs-25 C) of Cultures:					
	H-15	H-57	K162	202	217	224
None	-	+++	-	-	-	+++
.01% asparagine (aspn)	-	+	-	-	-	-
.02% aspn	-	-	-	-	-	-
.01% glutamine (gln)	-	+++	-	-	-	+++
.02% gln	-	+++	-	-	-	+++
.01% each of aspn and gln	-	+++	-	-	+	+++
.01% each of Aspn, gln and 5 amino acids[1]	+	+++	-	-	-	+++
.02% each of aspn, gln and 5 amino acids[1]	+++	+++	-	-	-	+++

1 = Arginine, leucine, isoleucine, methionine and valine

vals during 72 hrs incubation at room temperature.

The results showed that three cultures (8, 116 and 217) failed to grow, three (77, 160 and 188) grew poorly, one (43) grew fairly well and three (16, 18 and 201) grew very well.

It may be concluded from these data that certain strain of lactic streptococci require something more than the amino acids in casein or perhaps at different levels of concentration in the medium.

In a second trial four lots of agar medium were prepared. The nutrients in the medium were the same as in the first trial except that the adenine and uracil were replaced by 0.001% each of adenosine, guanosine, uridine and cytidylic acid. In order to eliminate the unnecessary amino acids, one lot of agar contained all 20 amino acids while the three additional lots contained progressively fewer acids as follows:

Lot No.	Amino Acids Omitted
1	None.
2	Group I: asparagine, aspartic acid, cysteine, glutamine and serine.
3	Group I plus Group II: glycine, threonine and tryptophan.
4	Group I and II plus Group III: alanine, lysine and tyrosine.

The concentrations of amino acids used were as follows, 0.005% each of cysteine and tryptophan, 0.01% each of arginine, glycine, histidine, methionine and phenylalanine, 0.015% each of alanine, isoleucine, proline, threonine, tyrosine and valine, 0.02% each of asparagine, aspartic acid, glutamine, leucine, lysine and serine and 0.025% glutamic acid. With the exceptions of glycine and dl-glutamine, the l-form of all amino acids were used. Six cultures of lactic streptococci and the auxano-

graphic method of measuring growth at 25° C during 60 hr incubation were used in this trial. The results are shown in Table II.

The results show that all six of the cultures grew well in the medium with the 20 amino acids added and in the medium (Lot 2) in which asparagine, aspartic acid, cysteine, glutamine and serine were omitted. The omission of glycine, threonine and tryptophan (Lot 3) and of alanine, lysine and tyrosine (Lot 4) resulted in good growth of all except culture N-49.

These results confirm the previous observation that different strains of lactic streptococci have different nutrient requirements. It appeared that either glycine, threonine or tryptophan was essential for culture N-49.

In the previous trial culture 217, in addition to others, failed to grow in the medium employed while in this second trial it grew very well. This indicates that the substitution of adenosine, guanosine, uridine and cytidylic acid improved the medium.

In a third trial the auxanographic method of measuring growth at 25°C during 60 hr incubation was used with the same cultures as in the second trial. The same basic medium was used except that nine amino acids that are considered to be essential (arginine, glutamic acid, histidine, isoleucine, leucine methionine, phenylalanine, proline and valine) were added. To portions of the medium were added six amino acids, (alanine, glycine, lysine, threonine, tryptophan and tyrosine) singly, and in various combinations as shown in Table III. The results show that five of the six cultures, 13, 17, 43, 201 and 217, grew well in the base medium containing the nine essential amino acids plus the nucleotide (cytidylic acid) and the nucleosides (adenosine, guanosine

TABLE II

AMINO ACID REQUIREMENTS FOR GROWTH OF LACTIC STREPTOCOCCI

Amino Acids Omitted	Relative Amount of Growth of Cultures:					
	13	17	43	201	217	N-49
None	++++	++++	++++	++++	++++	++++
Group I. Asparagine, aspartic acid, cysteine, glutamine and serine	++++	+++	+++	+++	+++	+++
Group I plus glycine, threonine and tryptohan (group II)	++++	+++	+++	+++	+++	-
Group I and II plus alanine, lysine and tyrosine	++++	+++	+++	+++	+++	-

Growth:
- = None
+ = Very Slight
++ = Fair
+++ = Good
++++ = Very Good

TABLE III

INFLUENCE OF ADDED AMINO ACIDS ON GROWTH OF LACTIC STREPTOCOCCI

Amino Acids Added	Relative Amount of Growth of Cultures:					
	13	17	43	201	217	N-49
None	++++	+++	+++	+++	+++	-
Glycine (gly)	++++	+++	+++	+++	+++	-
Threonine (thr)	++++	+++	+++	+++	+++	+
Tryptophan (try)	++++	+++	+++	+++	+++	+
Gly + thr	++++	+++	+++	+++	+++	+
Gly + try	++++	+++	+++	+++	+++	+
Thr + try	++++	+++	++++	+++	+++	++
Gly + thr + try (group II)	++++	+++	++++	+++	+++	++
Group II + Alanine (ala) + lysine (lys)	++++	+++	++++	+++	+++	++
Group II + Ala + tyrosine (tyr)	++++	++++	++++	++++	++++	++++

TABLE III (Continued)

Amino Acids Added	Relative Amount of Growth of Cultures:					
	13	17	43	201	217	N-49
Group II + lys + tyr	++++	++++	++++	+++	+++	+++

Growth:
- = None
+ = Very Slight
++ = Fair
+++ = Good
++++ = Very Good

and uridine) as sources of nitrogen. The remaining culture, N-49, grew very poorly in the medium. With this culture, some improvement in growth was observed when threonine and tryptophan with or without glycine were added and also when these three amino acids were in combination with alanine and lysine. Culture N-49 grew very well when combinations of glycine, threonine, tryptophan and tyrosine plus alanine or lysine were added to the base medium. It appeared that tryptophan and tyrosine were essential for growth of culture N-49 as very good growth occurred only when these two amino acids were present. Other possible essential amino acids were alanine and threonine.

In order to prove which amino acids are essential for the growth of certain strains of lactic streptococci, the previous trial was repeated except that alanine, threonine, tryptophan and tyrosine were added to the base medium singly and in all possible combinations. The cultures used were the same as in the previous trial plus three additional cultures (210, 218 and N-50). As in the previous trial the five cultures, 13, 17, 43, 201 and 217 plus one of the additional culture, N-50, grew very well on the base medium while the remaining three cultures, 210, 218 and N-49, grew very poorly. The results of these latter cultures are shown in Table IV.

The cultures grew very poorly when alanine, threonine and tryptophan were added singly or when a combination of alanine and threonine were added. The addition of tyrosine alone or in combination with alanine and/or threonine resulted in fair to good growth. Very good growth occurred with all three cultures when tryptophan and tyrosine were present. These results indicate that these latter amino acids are essential for certain strains of lactic streptococci. It may be noted

TABLE IV

INFLUENCE OF ADDED AMINO ACIDS ON GROWTH OF LACTIC STREPTOCOCCI

Amino Acids Added	Relative Amount of Growth of Cultures:		
	210	218	N-49
None	-	+	-
Alanine (ala)	-	+	-
Threonine (thr)	+	+	+
Tryptophan (try)	+	+	+
Tyrosine (tyr)	++	++	++
Ala + thr	-	+	+
Ala + try	+	++	+
Ala + tyr	++	+++	++
Thr + try	++	++	++
Thr + tyr	+	+++	+
Try + tyr	+++	+++	+++
Ala + thr + try	++	++	++
Ala + thr + tyr	+	+++	+
Ala + try + tyr	+++	+++	+++
Thr +try + tyr	+++	+++	+++
Ala + thr + try + tyr	++++	++++	++++

Growth:
- = None
+ = Very Slight
++ = Fair
+++ = Good
++++ = Very Good

that culture 218 appeared to be less fastidious than cultures 210 and N-49. Although these results appear to be conclusive, the possibility remains that certain additional strains of the lactic streptococci may exhibit different requirements for amino acids.

Mineral Requirements of Lactic Streptococci

Since minerals play important roles in the biochemical phenomena of cells an attempt was made to determine the mineral requirements of lactic streptococci. From the preceding trials it was found that 11 amino acids together with certain nucleosides, nucleotide, minerals and vitamins supported growth in all cultures tested. Therefore, in order to measure the mineral requirements for these organisms, a base medium was prepared in which the 11 amino acids found to be essential in the preceding section and adenosine, guanosine, uridine and cytidylic acid were used. The vitamins were added but no minerals were used. The medium was divided into portions, to one of which 1% of the mineral mixture (see methods) was added, while to the other portions were added 1% each of iron, magnesium, manganese and sodium chloride as aqueous solutions (see methods). One portion was left with no addative. Growth was measured by the auxanographic method after 48 hrs at 25 C. Six pure lactic streptococcus clutures, which had been propagated in basal Medium B broth (see methods) were used in this trial. The results, Table V, showed that three cultures, 43, 201 and N-49, grew well with no mineral supplement whereas the remaining three cultures, 13, 17 and 217, grew fairly well. The addition of Fe, Mg, Mn and NaCl individually or as a mixture of all four did not appear to affect the growth of the organisms. These results indicated that minerals might have been

carried over with the inoculum; as a result a second trial was conducted in the same manner as above except that the cultures were propagated once in the broth without any mineral supplement added. The results are presented in Table V.

It appeared that much less growth was obtained than in the first trial. Two cultures, 201 and N-49, grew only fairly well in the broth with no mineral supplements while the remaining cultures showed only slight growth. When a mixture of the four minerals was used, it appeared that all cultures grew slightly better than in the control plate with no minerals added. With the minerals added individually there appeared to be no marked improvement compared to the growth in the control plate.

The results of these two trials suggest that the technique used was unsatisfactory for determining the mineral requirements for these microorganisms. Previous work established that only trace amounts of minerals are needed (4 ppm or less). This fact together with the probability that some of the ingredients used in the medium employed contained minerals in trace amounts may account for the negative results obtained.

Medium for Routine Propagation of Lactic Streptococci

Niven (40) reported that hydrolyzed casein with other essential nutrients supported growth of 21 lactic streptococci only when the cooled, sterilized medium was fortified with glutamine and asparagine which were sterilized by filtration; however, Pollack and Lindner (53) demonstrated that a high level of glutamic acid would substitute for the filtered glutamine while asparagine was not necessary. In the

TABLE V

GROWTH RESPONSES OF LACTIC STREPTOCOCCI TO ADDED MINERALS

Mineral Added	Trial 1						Trial 2					
	Extent of Growth of Cultures:											
	13	17	43	201	217	N-49	13	17	43	201	217	N-49
None	++	++	+++	+++	++	+++	+	+	+	++	+	++
Fe	++	++	+++	+++	++	+++	+	++	++	++	+	++
Mg	++	++	+++	+++	++	+++	++	++	+	++	+	++
Mn	++	++	+++	+++	++	+++	++	++	++	++	+	++
NaCl	++	++	+++	+++	++	+++	+	++	+	++	+	++
All four	++	++	+++	+++	++	+++	++	++	+++	+++	++	+++

Growth:
- = None
+ = Very Slight
++ = Fair
+++ = Good
++++ = Very Good

development of a medium for routine propagation of lactic streptococci, it appeared necessary to determine whether it was essential to add filtered glutamine and asparagine to the medium. Medium A (see methods) was prepared. This medium with 0.01% sodium thioglycollate had that same formula as that used by Niven (40) except that the xanthine and guanine were omitted as they were not available and the concentrations of adenine and uracil were doubled. This change was justifiable from the report by Niven that any one of these four nucleic acid bases could be omitted without impairment of the medium.

The broth medium was divided into three portions: one with no glutamine and asparagine added, another with 0.01% each of glutamine and asparagine added before sterilization and a third portion with the glutamine and asparagine solutions sterilized by filtration and added to the sterile medium.

Twenty-four cultures were used in this trial and the amounts of growth was measured with the spectrophotometer at intervals during 72 hr incubation at 32 C. The results are shown in Table VI.

The results indicated that the addition of asparagine and glutamine before sterilization apparently had no effect on the growth of the cultures. When these materials were added after sterilization the growth of cultures 160, C_1, C_2 and N-49 appeared to be slightly better than the control without these materials added, while with cultures 17 and 173 the asparagine and glutamine appeared to be slightly inhibitory.

Of the 24 cultures used in this trial only seven grew very well (17, 18, 65, 135, 201, C_2 and N-49), six grew only fairly well (13, 16, 138, 160, 173 and 217) while the remaining 11 cultures failed to grow in the medium or grew very poorly. This indicates that the

TABLE VI

GROWTH OF LACTIC STREPTOCOCCI IN A MEDIUM CONTAINING HYDROLYZED CASEIN WITH OR WITHOUT GLUTAMINE AND ASPARAGINE

Culture Number	Optical Density		
	No Gln & Aspn	Gln & Aspn added before sterilization	Gln & Aspn added after sterilization
Control	.000	.000	.000
13	.168	.181	.155
16	.076	.092	.066
17	.208	.237	.125
18	.357	.357	.347
43	.018	.018	(contaminated)
65	.284	.284	.276
76	.009	.009	.013
77	.004	.009	.009
79	.004	.013	.013
116	.018	.013	.009
135	.357	.387	.367
138	.168	.187	.161
151	.009	.013	.013
160	.108	.131	.181
165	.018	.013	.018
173	.143	.168	.022
188	.004	.009	.013
191	.009	.013	.009
194	.009	.009	.004
201	.377	.409	.387
217	.155	.174	.137
C_1	.009	.009	.032
C_2	.377	.409	.482
N-49	.357	.420	.469

medium was nutritionally adequate for only a relatively few strains of lactic streptococci and that additional nutrients are needed.

Since the results obtained in the study of amino acids indicated that the replacement of the adenine and uracil by adenosine, guanosine, uridine and cytidylic acid markedly improved the medium, it was thought that these latter compounds might be valuable in a medium with hydrolyzed casein. Accordingly, Medium A used in the preceding experiment was modified by the elimination of the adenine and uracil and the addition of 0.001% each of adenosine, guanosine, uridine and cytidylic acid. This is designated as Medium B. Tubes of the sterilized broth were inoculated with 20 of the 24 cultures used in the preceding trial, incubated at 25°C for 21 hr and the amount of growth measured with the Klett-Summerson colorimeter. The results together with the optical density of growth obtained in the previous trial with Medium A (no asparagine or glutamine) are presented in Table VII.

Six cultures, 43, 76, 116, 165, 188 and 191, which grew poorly or failed to grow in Medium A employed in the previous trial, grew well in Medium B used in this trial. Likewise 4 cultures, 138, 160, 173 and 217, which grew fairly well on Medium A grew well in Medium B. Cultures 17, 18, 65, 135, 201, C_2 and N-49 grew very well in both media. The remaining 3 cultures grew fairly well in Medium B, two of which, namely 151 and 194, grew poorly or failed to grow in Medium A while culture 13 grew fairly well in both media. The results shows that Medium B containing hydrolyzed casein and fortified with adenosine, guanosine, uridine and cytidylic acid supported growth of all of the 20 cultures tested, with 17 of them growing very well and the remaining 3 fairly well in this medium.

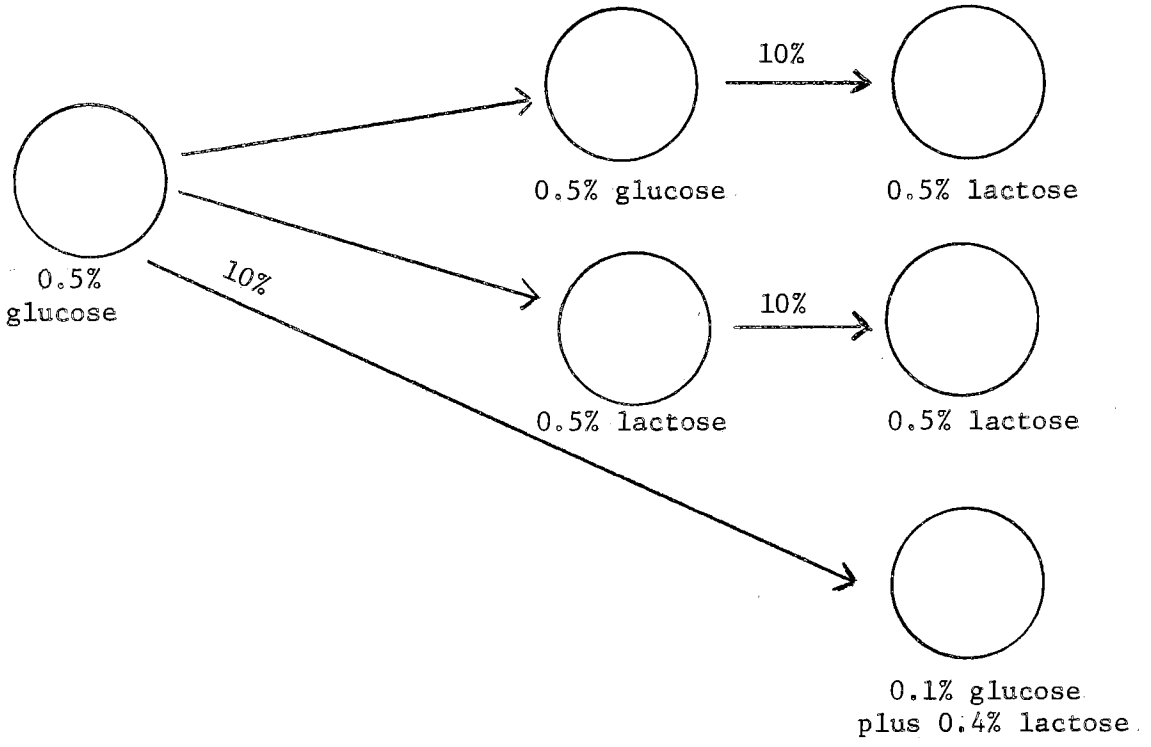
TABLE VII

THE INFLUENCE OF ADDING A NUCLEOTIDE AND NUCLEOSIDES TO HYDROLYZED CASEIN MEDIUM

Culture Number	Medium B with Adenosine, Guanosine Uridine and Cytidylic Acid	Medium A No Addatives
	Optical Density	
Control	.000	.000
13	.106	.168
17	.222	.208
18	.366	.357
43	.336	.018
65	.280	.284
76	.242	.009
116	.272	.018
135	.332	.357
138	.330	.168
151	.096	.009
160	.206	.108
165	.226	.018
173	.254	.143
188	.266	.004
191	.246	.009
194	.076	.009
201	.312	.377
217	.228	.155
C_2 N-49	.418	.377
	.392	.357

Induction of B-Galactosidase

To demonstrate that B-galactosidase is an inducible enzyme in lactic streptococci, two cultures were propagated in basal broth medium B (see methods) with 0.5% glucose as the carbohydrate source. The tubes were incubated until considerable growth had occurred as indicated by development of turbidity. The cultures were then inoculated into three tubes of the broth with (a) 0.5% glucose, (b) 0.5% lactose and (c) 0.1% glucose plus 0.4% lactose. After the cultures in tubes a and b had developed slight turbidity and presumably were in the beginning of the log phase of growth, they were inoculated at the rate of 10% into the broth with 0.5 lactose. The following diagram illustrates the steps involved with each cultures:



The increases in bacterial populations were measured periodically by determining the optical density with the spectrophotometer. The results are presented in Table VIII and shown graphically in Figures 1 and 2.

The results show that the cultures continued their logarithmic growth rates when transferred from lactose to lactose (Table VIII, Figure 1) while the cultures grown in glucose showed a lag phase when transferred into lactose (Figure 1). When the cultures propagated in glucose broth were then inoculated into broth containing both glucose and lactose, a similar lag phase was observed after glucose depletion (Figure 2). This lag period is the time required for the synthesis of B-galactosidase after induction by lactose. These results indicate that B-galactosidase is an inducible enzyme in lactic streptococci.

Effect of Certain Additives to Milk on the Rates of Acid Production by Lactic Streptococci

Since milk was not created as a medium for propagation of lactic streptococci, it was thought that perhaps certain additives to milk might improve the rate of acid production by these organisms.

Lactose is hydrolyzed by B-galactosidase (lactase) into galactose and glucose. Pyruvic acid is produced as an intermediate of glucose metabolism, which is in turn converted into lactic acid. Since lactic cultures use lactose to produce lactic acid, it seemed logical that the addition of substances produced during lactose metabolism might increase the rates of acid production by these organisms. It has been reported (13) that the addition of certain nucleosides stimulated acid production by lactic cultures. This together with the findings in the previous sections that the addition of certain nucleosides and nucleotide to a

TABLE VIII

INFLUENCE OF CARBOHYDRATE SOURCES IN THE INOCULUM AND IN THE TEST MEDIUM ON GROWTH RATES OF LACTIC STREPTOCOCCI

Time (hr)	Optical Density of Cultures: 43 glucose to lactose	43 lactose to lactose	43 glucose to glucose + lactose	217 glucose to lactose	217 lactose to lactose	217 glucose to glucose + lactose
0	.188	.173	.020	.142	.081	.010
0.5	.195	.200	.030	.144	.096	.010
1.0	.210	.235	.055	.150	.116	.013
1.5	.228	.270	.092	.155	.140	.020
2.0	.252	.305	.113	.162	.170	.032
2.5	.285	.312	.128	.168	.200	.052
3.0	.315	.315	.135	.178	.235	.061
3.5	.325	.312	.136	.185	.245	.065
4.0	.335	.310	.136	.195	.250	.074
4.5	.345	.312	.138	.214	.251	.069
5.0	.340	.308	.149	.235	.250	.074
5.5	.341	.310	.160	.250	.248	.083
6.0	.338	.308	.175	.260	.249	.088
6.5	.335	.308	.187	.263	.247	.098

Figure 1. Growth Curves of Lactic Streptococci in Medium Containing Lactose

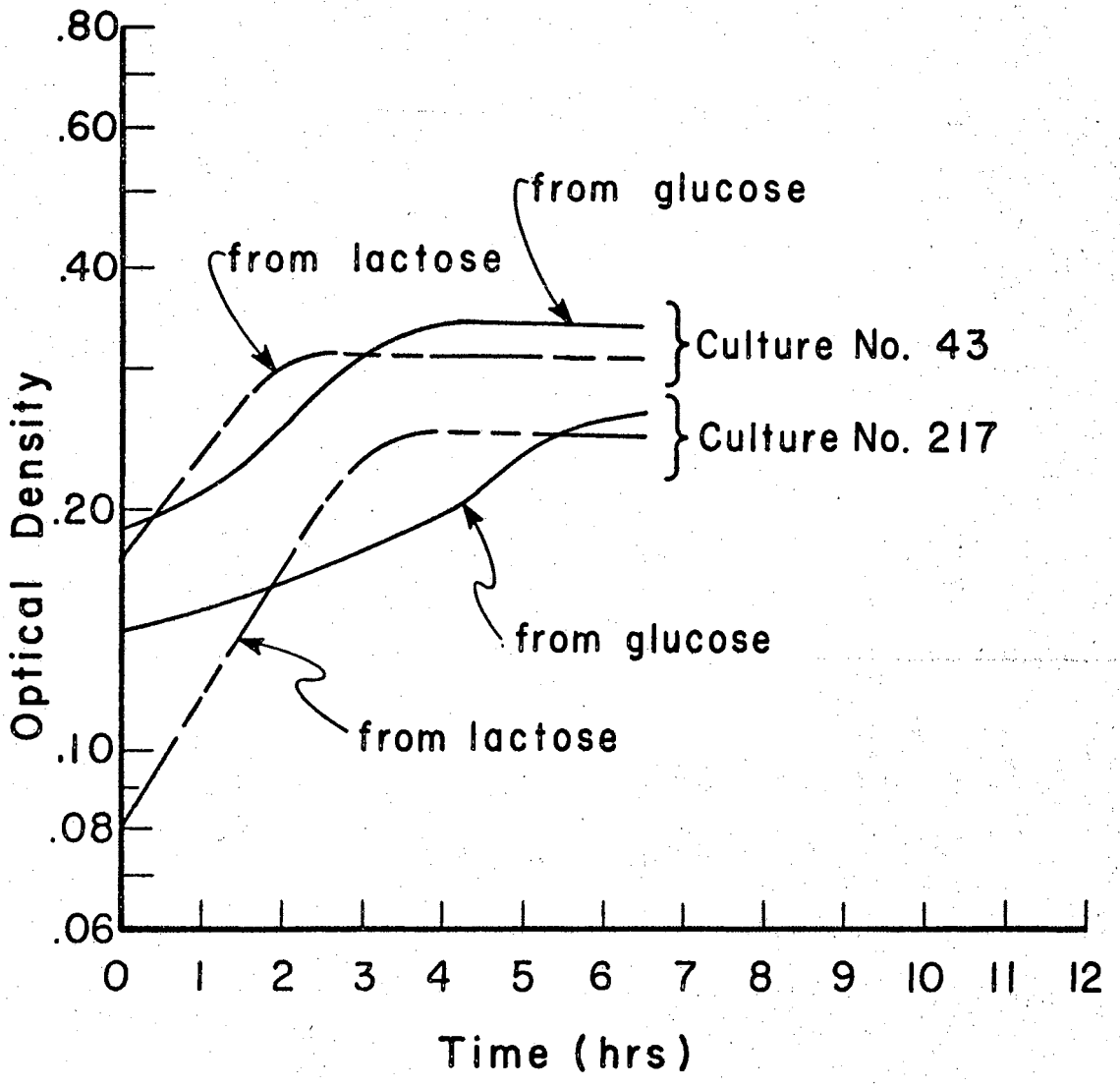
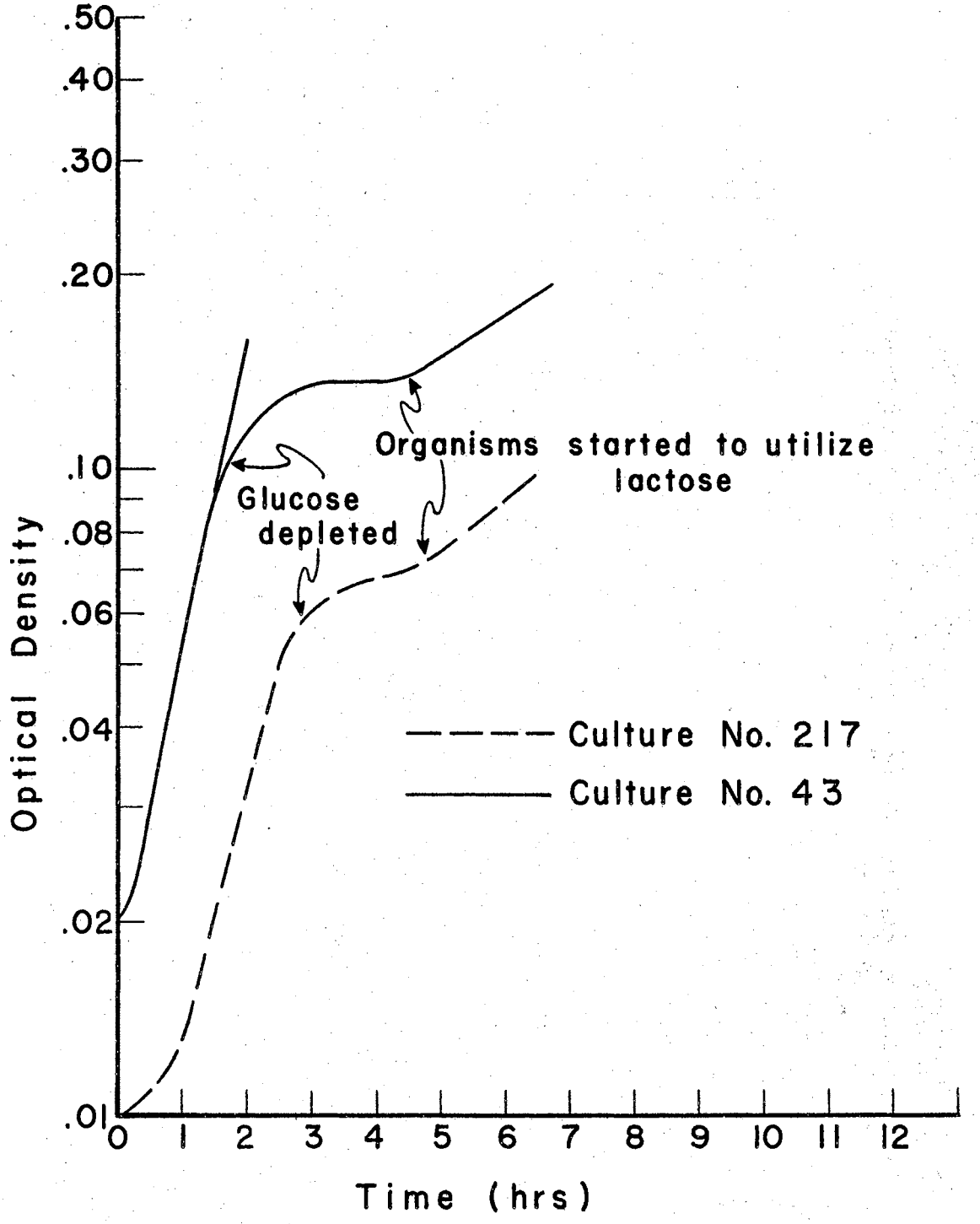

.80
.60
.40
.20
.10
.08
.06
Optical Density
from glucose
from lactose
Culture No. 43
Culture No. 217
from glucose
from lactose
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (hrs)

Figure 2. Growth Curves of Lactic Streptococci in Medium Containing both Glucose and Lactose


.50
.40
.30
.20
.10
.08
.06
.04
.02
.01
Optical Density
Glucose depleted
Organisms started to utilize lactose
Culture No. 217
Culture No. 43
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (hrs)

medium resulted in improved growth of lactic streptococci indicated that adding adenosine monophosphate (AMP) to milk might increase the rates of acid production by lactic streptococci.

Trials were conducted to determine the influence of adding AMP, glucose, galactose, pyruvic acid and the enzyme lactase on the rates of acid production in milk. The percentages of the materials added were as follows:

AMP	0.005, 0.01 and 0.02
Galactose	0.5, 1.0 and 2.0
Glucose	0.5, 1.0 and 2.0
Pyruvate	0.025
Lactase	0.005, 0.01 and 0.02

Separate trials were conducted for each addative. The responses to the addatives were measured by the four-hour activity test. The results are expressed in terms of the increases in titration values over those for the controls for the lots with the materials added. Only the values for what appeared to be the optimum concentrations are shown in the results in Table IX.

Observations on Additives

AMP. The addition of AMP to milk resulted in slight increase of acid production by the two cultures tested. The optimal level of AMP was near 0.01%, with 0.005% showing no increase and 0.02% only slight.

Galactose. Acid production by all four cultures used was stimulated by galactose. Furthermore, the 1.0% level of galactose appeared to be the optimum, with the level of 0.5% showing some stimulation and 2.0% no better than 1.0%.

TABLE IX

EFFECT OF CERTAIN ADDITIVES TO MILK ON THE RATES OF ACID PRODUCTION BY LACTIC STREPTOCOCCI

Culture Number	Increases in Titration Values (ml) Due to Adding:				
	.01% AMP	1.0% Galactose	1.0% Glucose	0.025% Pyruvate	.02% Lactase
43	.28	.20	.28	1.60	.70
201		.40	.65		
217	.10	.30	.68	.78	.40
N-49		.28	.35	.80	

Glucose. Glucose stimulated acid production by all four cultures with two of them showing rather larger increases. It was noted that the 1.0% level of concentration was near the optimum, with two of the cultures showing considerable stimulation with 0.5% added. The concentration of 2.0% showed less stimulation than 1.0%.

Pyruvate. Tremendous response in acid production by all three cultures was observed upon addition of 0.025% pyruvate to milk. Since only one level of concentration was used, the possibility exists that higher levels may have caused even greater responses.

Lactase. Lactase stimulated acid production by the two cultures tested. It was noted that acid production increased as the lactase concentration increased, suggesting that the optimal level had not been reached.

The results of the preceding trials showed that the addition to milk of AMP increased acid production by the cultures tested only slightly, while the addition of galactose resulted in slightly better increases than AMP. The addition of glucose resulted in even greater responses in acid production than either AMP or galactose. Furthermore, pyruvate addition resulted in a much greater increase in acid production than AMP, galactose or glucose. On the other hand, as level of lactase increased the rate of acid production increased. Had higher concentrations of the enzyme been used, the acid production might have increased.

Selection of Mutants of Lactic Streptococci

Selection of Mutants by the Use of a Chemostat

From a pilot test and from the work of others (9, 10) it was found that B-galactosidase has an inducible system which perhaps resembles that of E. coli. Horiuchi et al. (19), using the chemostat, obtained the so-called hyper strains of E. coli which are capable of synthesizing B-galactosidase in as much as 25% of total cell protein. This together with the findings that lactase increased acid production by the lactic streptococci tested and supported by the results of Woodard et al. (75), that B-galactosidase stimulated acid production by these organisms indicated that perhaps selection of lactic streptococcus mutants could be accomplished by using a chemostat. The principle in using the chemostat is to use a limited amount of one of the growth factors in the ingredients of the medium.

To determine the minimum concentration of lactose required to support growth of lactic streptococci, various levels of lactose were added to the base Medium B and the relative amounts of growth determined with the spectrophotometer. The lactose was added at levels of 0.5, 0.3, 0.2, 0.1, 0.05, 0.03, 0.02, 0.01 and 0.005%. Five pure cultures were used for inoculating the tubes which were incubated at 25 C for 24 hr before measuring the growth responses.

The results indicated that the minimum concentration for growth appeared to be at 0.02 to 0.03%. Consequently, the level of 0.03% was used in later trials with a chemostat.

Four preliminary trials were conducted in which Medium B containing 0.03% lactose was used as the nutrient medium. The chemostat was oper-

ated at room temperature and samples of the effluent were taken daily, inoculated into sterile litmus milk and rates of acid production determined by the four-hour activity test. Three cultures were used: N-49 in Trial 1, 43 in Trials 2 and 3 and 217 in Trial 4. The inflow rates were 10% in Trial 1 and 2 and 20% in Trial 4, while in Trial 3 it was 4% initially, then increased to 8% after 4 days and to 12% after 8 days. The results are presented in Table X.

The data showed that there appeared to be no significant increases in activity of cultures N-49 and 43, while with culture 217, which was more active than the other two, there appeared a slight increase after three days operation.

It should be mentioned that Trial 2 was conducted for three and Trial 3 for seven more days than shown but that heavy contamination in the medium nullified the results.

Furthermore, assays for B-galactosidase were conducted in cultures from the effluent but the results were negative or only slightly positive.

Several attempts were made to assay for B-galactosidase production by lactic streptococci growing in Medium B with 0.5 to 0.7% lactose. It was noted that among five cultures tested only culture 43 produced B-galactosidase stable enough to withstand the assay procedure. This is in general agreement with the findings of Cetti et al. (9, 10). As a result, culture 43 was used in some of the following trials. In addition culture 217 was used in one trial since it suggested an increase in rates of acid production during propagation in the chemostat.

Two trials were conducted using culture 43, in the chemostat in the same manner as in the four preliminary trials. In Trial 1 the inflow

TABLE X

ACTIVITY TESTS ON EFFLUENT SAMPLES FROM CHEMOSTAT PROPAGATED IN MILK

Period of Operation (Days)	Trial 1 Culture N-49	Trial 2 Culture 43	Trial 3 Culture 43	Trial 4 Culture 217
	Titration Values (ml)			
Control	5.9	5.7	6.1	6.6
0	6.1	5.5	6.3	6.8
1	5.8	5.6	6.3	6.1
2	5.9	5.3	6.3	6.6
3	6.0	5.2	6.5	7.0
4	6.4	5.4	6.3	7.2
5	6.1	5.8	6.4	7.2
6		5.9	6.1	7.0
7		5.9	6.2	
8		6.2	6.1	
9		6.0	6.3	

rate was increased to 15% after one day operation at 12%. While in Trial 2 it was initially at 15% then increased to 20% after one day and to 22% after two and a half days of operation. Samples of effluent were taken daily in Trial 1 and at half-day intervals in Trial 2, inoculated into sterile litmus milk and rates of acid production determined by the four-hour activity test. Each sample of effluent was also propagated in Medium B with 0.5% maltose then in milk and the activities determined. Furthermore, the maltose broth was assayed for B-galactosidase. The results are shown in Table XI.

The results indicate that acid production was increased by the culture in both Trial 1 and 2 after four and three days of operation respectively. The results also indicate that the increases in the rates of acid production were accompanied by corresponding increases in the level of B-galactosidase. Since this enzyme is inducible in lactic streptococci and the fact that a higher level of it was observed in the absence of an inducer, i.e. in the maltose broth, indicate that B-galactosidase constitutive mutants appeared to be present in those portions of the broth with high levels of the enzyme. It was also noted that the sample collected on the fourth day (Trial 2) and propagated in litmus milk showed low acid production compared to the amount of the enzyme produced. This indicates that the mutants in that portion were not stable. Trial 2 was conducted 5 more days than shown, but contamination was apparent after replenishment of the medium in the reservoir and the subsequent samples were apparently not valid.

Since it has been reported (44) that certain nucleosides supress the rate of mutation by E. coli, it was thought the omission of the nucleosides and nucleotide from Medium B might improve the rate of

TABLE XI

ACTIVITY TESTS AND β-GALACTOSIDASE ASSAYS ON EFFLUENT SAMPLES FROM CHEMOSTAT

(Culture 43)

Period of Operation (Days)	Trial 1			Trial 2		
	Titration Values (ml) When Propagated In		Enzyme* Assay In	Titration Values (ml) When Propagated In		Enzyme* Assay In
	Milk	Maltose then in Milk	Maltose Broth	Milk	Maltose then in Milk	Maltose Broth
Control	6.2	6.3	.41	6.3	6.3	.42
0	6.3	6.4	.41	6.3	6.3	.43
0.5				6.5	6.5	.41
1.0	6.0	6.1	.41	5.9	5.8	.42
1.5				6.2	6.3	.42
2.0	6.3	6.4	.42	6.2	6.2	.43
2.5				6.4	6.5	.42
3.0	6.6	6.5	.42	6.8	6.8	.59
3.5				7.3	7.2	.66

TABLE XI (Continued)

Period of Operation (Days)	Trial 1			Trial 2		
	Titration Values (ml) When Propagated In		Enzyme* Assay In	Titration Values (ml) When Propagated In		Enzyme* Assay In
	Milk	Maltose then in Milk	Maltose Broth	Milk	Maltose then in Milk	Maltose Broth
4.0	6.9	6.9	.54	6.3	7.1	.65
4.5				7.0	7.1	.67
5.0	7.2	7.3	.65	6.5	6.6	.62
5.5				6.4	6.5	.62
6.0	7.2	7.2	.65	6.3	6.2	.62

* $\frac{\text{Assay reading}}{\text{Turbidity reading}}$

mutation. Accordingly, Medium B was prepared with the nucleosides and the nucleotide omitted and with 0.4% yeast extract added. Culture 43 was used in this trial in which the rate of inflow was initially 20%, then increased to 40% after one day and to 60% after three days. The data, (Table XII), showed that the modified Medium B used in this trial gave results very similar to those obtained in the previous trials with Medium B and there appeared to be no advantage in substituting yeast extract for the nucleosides.

Culture 217 was used in a trial in which the rate of inflow was increased at intervals during 12 days of operation, starting with a 20% rate and increasing to a 90% rate for the last 3.5 days of the trial. The effluent was sampled at half-day intervals, inoculated into litmus milk and rate of acid production determined by the four-hour activity test. The samples were propagated two more times in milk and activities determined. Each sample of effluent was also propagated in Medium B with 0.5% maltose and then propagated in milk and the activities determined.

The data presented in Table XIII clearly indicate that, on the first propagation in milk, as the rate of inflow was increased the culture become more active, with the highest rates of acid production occurring when the inflow rate was 70%. The average titration values for the rates of inflow were as follows: 20%, 6.15; 30%, 6.15; 40%, 6.28; 50%, 6.45; 70%, 7.82 and 90%, 7.56. This latter value was for the first five samples taken after changing to the 90% rate. The subsequent values were considered erroneous because of the contamination that appeared at that time.

The second and third propagations in milk showed somewhat lower

TABLE XII

EFFECT OF NUCLEOSIDES OMISSION FROM THE CHEMOSTAT MEDIUM ON THE ACTIVITY TESTS AND B-GALACTOSIDASE ASSAYS

(Culture 43)

Period of Operation (Days)	Inflow %	Titration Values (ml) When Propagated in		Enzyme Assay in
		Milk	Maltose then in Milk	Maltose
Control		6.1	6.3	.42
0	20	6.3	6.7	.43
1	40	5.9	6.2	.43
2		6.2	6.8	.47
3	60	6.5	7.2	.56
4		5.8	6.6	.61
5		6.6	7.0	.65
6		6.7	7.2	.64
7		6.8	7.2	.65
8		6.9	6.5	.64

TABLE XIII

ACTIVITY TESTS ON EFFLUENT SAMPLES FROM CHEMOSTAT PROPAGATED IN MILK AND IN MALTOSE BROTH

(Culture 217)

Period of Operation (Days)	Inflow %	Propagations in Milk			Propagated in Maltose then in Milk
		First	Second	Third	
		Titration Values (ml)			
Control	--	6.3	6.2	6.3	6.2
0	20	6.1	6.2	6.1	6.3
0.5		6.2	6.3	6.4	6.3
1.0	30	6.4	6.7	6.6	6.4
1.5		5.9	6.1	6.3	6.2
2.0	40	6.3	6.8	6.4	5.9
2.5		5.9	6.1	6.2	6.2
3.0		6.2	6.3	6.6	6.4
3.5		6.7	7.0	6.7	6.2
4.0	50	6.7	7.1	6.8	6.7
4.5		6.2	6.5	6.6	6.3
5.0	60	6.7	6.4	6.8	6.6
5.5		6.5	6.0	6.4	6.2
6.0	70	7.3	6.9	7.2	6.6
6.5		7.6	6.7	6.8	6.4
7.0		8.0	7.4	7.1	6.8
7.5		8.1	7.3	7.0	6.8
8.0		8.1	7.5	7.2	6.7
8.5	90	8.0	7.4	7.0	6.8
9.0		7.6	7.0	6.7	6.5
9.5		7.5	6.5	6.9	6.1

TABLE XIII (Continued)

Period of Operation (Days)	Inflow %	Propagations in Milk			Propagated in Maltose then in Milk
		First	Second	Third	
		Titration Values (ml)			
10		7.6	6.6	6.5	6.2
10.5		7.1	6.6	6.4	6.1
11		6.0	6.4	5.9	6.0
11.5		5.8	5.9	5.5	5.9
12		5.4	5.6	5.5	5.9

rates of acid production than the first propagations but the most active subcultures were in the range considered to be very satisfactory for cheesemaking. It was also noted that when the samples were propagated in the maltose broth before subculturing in litmus milk the rates of acid production were lower than when the effluent samples were inoculated directly into litmus milk.

Wilkowske and Fouts (71) attempted to develop a continuous process for propagation of lactic cultures (S. lactis and Leuconostoc species) and succeeded for periods up to three weeks before contaminations became dominant in the cultures. It was presumed that, similarly, the Chemostat could be used with milk as the medium to develop and maintain high rates of acid production by pure cultures of lactic streptococci.

One trial was conducted in which skimmilk, adjusted to a pH of 7.5 with sodium hydroxide and sterilized, was used as the growth medium in the Chemostat. Culture N-49 was used and the Chemostat operated at room temperature (25 C) for 18 days. The inflow rate was 40% during the first five days and 60% for the last 13 days. Samples of the effluent were taken daily, and activity tests conducted on the first and second propagations in litmus milk. The results are presented in Table XIV. Since the changes that occurred in rates of acid production were rather gradual, the average titration values for successive days are shown in order to conserve space.

The data on the first propagation show that the rates of acid production increased during the first five days of operation at an inflow rate of 40%, then decreased during the next five days after the rate was increased to 60% followed by a progressive increase in activity during the next seven days.

TABLE XIV

ACTIVITY TESTS ON EFFLUENT SAMPLES FROM CHEMOSTAT USING SKIMMILK AS THE PROPAGATING MEDIUM

Period of Operation (Days)	Titration Values (ml) After Propagation	
	First	Second
0	6.3	5.6
0-2	6.5	5.9
3-4	6.6	6.0
5-6	6.6	6.1
7-8	6.3	6.1
8-9	6.1	6.2
10-11	6.4	6.4
12-13	6.7	6.8
14-15	6.7	6.4
16-17	6.9	7.7
18	6.2	5.6

Contamination was evident at this point which probably accounted for the low value obtained on the 18th day.

The titration values for the second propagations followed the same general pattern except that all the values were rather low during the first nine days of operation. The increases in activity from the 11th through the 17th day were generally greater than those obtained on the first propagations.

Some trouble was experienced by coagulation clogging the effluent tube. This perhaps could have been avoided by maintaining the pH in the growth chamber at a predetermined level by adding sodium hydroxide or other neutralizer.

The results indicate that the rate of acid production may be increased substantially by continuous propagation and that such a procedure might be of practical value in a cheese plant.

Mutagenesis By NTG

Selection for mutants by the use of the Chemostat is rather a slow process, therefore, an attempt was made to mutagenize lactic streptococci by N-methyl-N-nitro-N-nitrosoguanidine (NTG) using the Adelberg method (1), with slight modification. Three trials were conducted.

In the first trial culture 43 was propagated in Medium B containing 0.5% galactose, mutagenized, with NTG and diluted 1:1000 and 0.1 ml portions of diluted cell suspension were inoculated into each of 20 test tubes containing glucose broth. After incubation a portion of each tube was assayed for B-galactosidase. The culture in each tube was then inoculated into sterile litmus milk and the activity determined. Also, each tube was inoculated into galactose broth and, after growth, assayed

for the enzyme.

In the second trial lactose broth was used for growing the culture for mutagen treatment. The treated and diluted cell suspensions were inoculated into maltose broth for enzyme assay and also directly into litmus milk for activity tests. In addition the cultures in maltose broth were propagated in litmus milk for activity tests.

The results of the two trials are shown in Table XV. In this Table the enzyme assay (in glucose) values for the 20 subcultures are arranged in ascending orders to facilitate observations on the enzyme production and activity tests.

The data in Trial 1 show that there was a general relationship between B-galactosidase production and rate of acid production. The last four subcultures listed in the Table show very high values for enzyme production and very high values for rates of acid production. These values are outstanding when compared to those for the control indicating, that mutation had been effected by the treatment with NTG. The results also indicate that these mutants are constitative for making B-galactosidase since they produce the enzyme in the absence of an inducer. Furthermore, when the glucose broth subcultures were propagated in galactose broth enzyme production appeared to be much higher than in the glucose broth. Induction by galactose could not have accounted for the increase in the amount of the enzyme since the control, treated under the same condition, showed very low amount of enzyme production.

The results for the second trial show the same general relationship between enzyme production in maltose broth and rate of lactic acid production in milk; however, it appeared that the minimum values were higher and the maximum values for enzyme production lower than those obtained

TABLE XV

INFLUENCE OF TREATMENT WITH MUTAGEN AGENT (NTG) ON B-GALACTOSIDASE PRODUCTION AND ACTIVITY OF A LACTIC STREPTOCOCCUS

(Culture 43)

Trial 1			Trial 2	
Titration Values Propagated in Glucose then Milk	Enzyme Assay of Treated Cells* Propagated in Glucose Broth	Enzyme Assay of Treated Cells* Propagated in Glucose then Galactose	Titration Values Propagated in Maltose then Milk	Enzyme Assay* in Maltose Broth
6.0**	.40**	.29**	6.0**	.42**
5.8	.39	2.48	6.2	.46
6.2	.42	1.80	5.8	.47
6.3	.44	1.29	6.1	.47
6.2	.44	1.41	6.2	.47
6.5	.45	1.08	6.2	.48
6.4	.45	1.12	6.4	.48
6.6	.46	1.20	6.2	.51
6.6	.46	1.24	6.4	.51
6.5	.51	1.25	6.2	.53
6.6	.53	1.20	6.4	.53
6.6	.53	1.58	6.3	.54
6.8	.59	1.59	6.2	.55
6.7	.62	1.67	6.4	.56
6.7	.63	1.49	6.7	.57
6.7	.63	1.66	6.8	.58
7.6	.85	1.59	7.3	.62

TABLE XV (Continued)

Trial 1			Trial 2	
Titration Values Propagated in Glucose then Milk	Enzyme Assay of Treated Cells* Propagated in		Titration Values Propagated in Maltose then Milk	Enzyme Assay* in Maltose Broth
	Glucose Broth	Glucose then Galactose		
7.4	.91	1.50	7.3	.63
7.8	1.01	1.88	7.5	.63
7.9	1.16	1.84	7.6	.66

* $\frac{\text{Assay reading}}{\text{Turbidity reading}}$

** Control Culture - Not Treated

in the first trial.

In the third trial an attempt was made to determine the proportion of slow and fast strains of lactic streptococci among the isolates from mutagenized cell suspension. In this trial cottage cheese whey was used as medium for growth hoping to get large proportion of fast strain since rapid acid production in cheese is one of the prime objectives of this research. Cheese whey was neutralized to pH 6.5, heated in flowing steam to coagulate albumin, filtered, dispensed in test tubes and sterilized. The whey was inoculated with culture 18 and, after considerable growth occurred, the cells were mutagenised as before. The cell suspension was streaked over Medium B agar containing 0.5% lactose. After incubation colonies were picked and propagated in sterile litmus milk for activity. Selected cultures were propagated a second time in milk and tested for activity. A summary of the activity tests and activity ratings of 58 cultures are presented in Table XVI.

The results show that 10 of the 58 cultures were very slow cultures since they had titration values below 6.0. Furthermore, two of the ten cultures had titration values of 2.3 ml indicating that undesirable mutation (noninducible) had occurred. Ten cultures were found to be slow since their titration values were in the range of 6.1 to 6.5. Sixteen cultures had titration values between 6.6 and 7.0 and were considered as satisfactory for use in cheese making. The remaining 22 cultures had titration values greater than 7.1 and were considered very fast. Since 38 of the 58 cultures isolated (65.5%) were considered satisfactory or showed very rapid acid production, it appeared that treatment of the cells with the mutagen was beneficial; however, it should be noted that 10 of the isolates were very slow, which indicates that the mutagen had

TABLE XVI

ACITIVTY RATINGS OF PURE CULTURES ISOLATED FROM A MUTAGENIZED WHEY CULTURE (Culture 18)

Number of Cultures	Range in Titration Values (ml)	Activity Rating
10	<6.0	Very Slow
10	6.1-6.5	Slow
16	6.6-7.0	Satisfacotry
22	7.1-7.5	Fast

an adverse effect on these strains.

Twenty-eight of the cultures were propagated in milk a second time and activity tests conducted. The activity tests on the second propagations were essentially the same as those on the first propagation except in two instances. One of these showed a decrease in titration value of 0.7 ml while the other showed an increase of 0.7 ml.

CHAPTER V

SUMMARY AND CONCLUSIONS

In order to determine the mineral requirements of lactic streptococci, it was necessary to develop a chemically defined medium. Media containing different sources of nitrogen compounds, glucose and other essential factors were prepared. The results showed that ammonium sulfate and urea plus seven amino acids as sources of nitrogen for lactic streptococci were inadequate. The replacement of adenine and uracil by adenosine, guanosine, uridine and cytidylic acid in a medium containing the 20 amino acids naturally occurring in proteins, glucose, a buffer, vitamins and minerals improved the medium which otherwise was inadequate for some strains. By omission of groups of amino acids from the improved chemically defined medium and readdition to the same of certain amino acids singly and in combination with each others, nine amino acids were eliminated without impairment of the medium. It was concluded that the remaining 11 amino acids (arginine, glutamic acid, histidine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine) were essential for growth of lactic streptococci. When the chemically defined medium without minerals added was used in an attempt to determine the mineral requirements of lactic streptococci, growth was observed in the absence of the minerals tested (Fe, Mg, Mn, NaCl). It was apparent that some or perhaps all of the ingredients of the medium contained traces of minerals and rendered it ineffective for this purpose.

In order to avoid the hazard from the occasional presence of antibacterial substances and the high calcium content of milk which is favorable for bacteriophage adsorption, a medium was developed for routine propagation of lactic streptococci. A medium containing a sugar, hydrolyzed casein, adenosine, guanosine, uridine and cytidylic acid plus a buffer, vitamins and minerals was found to be adequate.

Since glucose, galactose and pyruvate are produced during the conversion of lactose to lactic acid by B-galactosidase (lactase) and since certain nucleosides stimulated acid production by lactic streptococci it was thought that the addition of these materials to milk might be effective in increasing the acid production. It was found that the addition of adenosine monophosphate, galactose, glucose, lactase and pyruvate were effective in increasing the rates of acid production in the order listed. Although pyruvate appeared to give the highest rate of acid production, the optimum concentration of lactase may not have been attained.

Since B-galactosidase is an inducible enzyme in lactic streptococci it was thought that mutants capable of high rates of acid production could be produced by the use of a continuous flow apparatus (chemostat) and a limited amount of lactose in the medium. Broth containing 0.03% lactose was pumped into the growth chamber at flow rates ranging from 4 to 90%, and samples of the effluent were taken at intervals for activity tests. In the several trials conducted it was found that increases in acid production were observed at the low flow rates but that pronounced increases occurred when the flow rate was increased stepwise to 70%. At the 90% rate of inflow it appeared that reduction in population in the growth chamber resulted in somewhat lower rates of acid production. It

was concluded that the continuous flow apparatus was selective for mutants with high growth rates which are related to high rates of acid production. Similar but less pronounced increases in rates of acid production were obtained when skimmilk was used as the growth medium.

Three trials were conducted in attempts to develop mutants with rapid acid production by treatment with the mutagen N-methyl-nitro-N-nitrosoguanidine (NTG). Cell suspensions of the cultures were treated with the mutagen, 20 portions were inoculated into separate tubes of broth and, after propagation, the cultures tested for B-galactosidase and for rate of acid production after propagation in litmus milk. The results indicated that the NTG mutagen caused mutation, with the acid production rates of the mutants ranging from slow to very fast. Assays for B-galactosidase showed that there was a general relationship between the amounts of the enzyme produced and the rates of acid production by the cultures. In one of the trials the mutagenized cell suspension was streaked on agar and 58 pure cultures isolated. Activity tests were conducted on the cultures isolated. Of these, 38 (65.5%) were classified as fast, or as satisfactory for cheesemaking, while the remaining cultures were classified as slow or very slow. The results indicate that a mutagen may be used to induce mutants capable of producing acid at a high rate.

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