INVESTIGATIONS OF THE SODIUM AND POTASSIUM REQUIREMENTS OF LACTIC ACID BACTERIA

By

JOHN NORMAN MILLS

Bachelor of Arts

Oshkosh State College

Oshkosh, Wisconsin

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Thesis Approved:

Robert J. Lacey
Thesis Adviser

Robert MacVicar

Robert MacVicar
Dean of the Graduate School

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GENERAL INTRODUCTION

The essence of a statement made by I. S. Falk (1) in 1923,

> "The basis on which rests the use of synthetic media is still essentially emperical. The physiological significance of this or that ion is not known and cations or anions for that matter are thrown in or taken out of media according to whether growth is good, bad or indifferent",

is still fundamentally true today, more than thirty years later. Until the last decade the idea was generally accepted that 'whether growth was good, bad or indifferent' depended only upon the ratio of the minerals present. No attempts were made to relate ion interrelationships to the nutritional requirements of the organisms studied. In 1912 Loeb (2) attributed the total effect of neutral salts on life preservation to their beneficial effect on the permeability of the cell wall providing these salts were present in certain appropriate ratios. Using similar reasoning Falk (3), in 1922 stated that "the effect exerted by electrolytes appears to be primarily an effect upon the external or internal membrane surfaces and upon surface interphases in colloidal systems".

During the 1940's when the mineral requirements of lactic acid bacteria were studied extensively by such workers as MacLeod and Snell (4) it was found that there existed not only a requirement for such minerals as potassium, manganese and phosphorus but also that these requirements were not

absolute. These workers noted that these requirements were often too large to be explained only by mineral utilization in direct metabolic processes. Further investigations revealed that there was a sparing effect by some minerals on the requirements of certain other minerals. This is illustrated by the sparing effect of magnesium, strontium or calcium on the manganese requirement of Lactobacillus arabinosus, as shown by MacLeod and Snell (5). They suggested that the sparing effect of an ion depended upon the number of enzyme systems, activated by the essential ions, which may also be activated by the sparing ion. Similar reasoning suggests that the degree of antagonism due to one mineral ion depends upon the number of enzyme systems activated by an essential ion which is antagonized by the other ion.

The lack of a clear understanding of the specific roles of these minerals, especially because of the importance of these organisms in analytical determinations and in elucidations of metabolic pathways, justifies extensive investigation of this aspect of their nutrition. A prerequisite for these investigations is an evaluation of the known roles of organic substances in their nutrition. A number of reviews(6-9) have appeared concerning our present knowledge of the nutrition of the lactic acid bacteria, and an evaluation of this knowledge is presented in the remaining paragraphs of this general introduction.

The family Lactobacteriaceae is characterized by its ability to ferment sugars with the production of considerable

quantities of lactic acid. Homofermentative forms produce an 80-98% yield of lactic acid whereas heterofermentative forms produce about a 45% yield and also produce CO_2, H_2, alcohol, formic and acetic acid from the sugar. All evidence indicates that this lactic acid is formed by reactions of the Embden-Myerhof scheme. The main sources of energy for these micro-organisms are soluble carbohydrates.

Ingredients of the media which may be directly incorporated into the cellular protoplasm include amino acids. The ability of lactic acid bacteria to synthesize amino acids is very limited as indicated by the relatively high number of amino acids which are essential for their growth. Leuconostoc mesenteroides, for example, requires seventeen of the eighteen common amino acids. Other species are less fastidious but their ability to synthesize certain amino acids depends not only on such factors as other constituents and pH of the medium, but also on the concentrations of other amino acids in the medium. These amino acid interrelationships in lactic acid bacteria have revealed and will undoubtedly continue to reveal fundamental information of incalculable value for understanding the nutrition of higher forms of life and also for improving the usefullness of lactic acid bacteria as analytical tools.

As indicated above the requirements for vitamins are closely related to the amino acid content of the media. They are also related to the purines and pyrimidines present which serve as precursors for the synthesis of bacterial nucleic acids.

An interrelationship between a vitamin and an amino acid is illustrated by the relativily high biotin requirement of Lactobacillus arabinosus in media lacking aspartic acid (10). Tracer experiments have confirmed the role of biotin in the synthesis of aspartic acid (11). Similarily, under certain conditions, the pyrimidine thymidine can replace the citrovorum factor required by Leuconostoc citrovorum (12).

Thus, the complexity of the interrelationships among various organic nutrients is obvious. The situation is further complicated by the fact that metabolism of organic nutrients is altered in various ways by inorganic substances. All of these interrelationships are regulated by such further interrelated factors as the hydrogen ion concentration, the carbon dioxide tension and the temperature of incubation.

The present study was designed to evaluate more thoroughly these interrelationships or factors affecting the known potassium requirement of lactic acid bacteria and to apply the information gained about these factors in attempts to demonstrate a possible sodium requirement in these bacteria.

Following a discussion of the general procedures used, the results of these studies will be presented in two main parts. The first will consist of a detailed consideration of the effects of pH on the potassium requirement of certain lactic acid bacteria. The second part will present studies on a possible sodium requirement, with an emphasis on the factors affecting the requirement of a trace mineral.

GENERAL PROCEDURES

Assay Organisms:

These studies were made with the following strains of lactic acid bacteria which are representative of those commonly used in microbiological assay work:

Leuconostoc mesenteroides P-60 (ATCC 8042)

Lactobacillus arabionsus 17-5 (ATCC 8014)

Lactobacillus delbruckii (L. d. III Henneberg)[1]

Streptococcus faecalis R (ATCC 8043)

Twelve to eighteen hours prior to use as inocula, transfers of the appropriate organisms were made to 2 ml. of a liquid transfer medium (Appendix A). These broth cultures were then incubated at 37°C and inocula were prepared from these by centrifugation of the cells and resuspension in 25 ml. of glass - distilled water. A drop of this suspension was added to each assay tube with the aid of a syringe.

Basal Medium:

The concentrations of the organic nutrients and inorganic ions were essentially the same as those recommended by Henderson and Snell (13), with appropriate modifications. It should be realized that these nutrients added as part of the basal medium are present in excessive amounts to minimize effects produced,

[1]Described by Sirny et al. in J. Nutrition, 41:383, (1950).

as a result of varying assay conditions, by nutrients other than those being studied.

Many different modifications of this basal medium were used in these studies, and specific descriptions will be presented subsequently. However, media with these various modifications may be classified into 3 main types, based on their variation in sodium and/or potassium content, as follows: (1) the Na-K medium which is the Henderson-Snell medium essentially as described (Appendix B), (2) the all-Na medium in which all potassium salts are replaced by equimolar amounts of corresponding sodium salts, and (3) the all-K medium in which all of the sodium salts are replaced by potassium salts. The hydrogen - ion concentrations of the all-K and Na-K media were adjusted by the addition of KOH to the originally-acidic media; the pH of the all-Na media was similarily adjusted by the addition of NaOH. When more than one pH was studied in a single assay, KCl was added to the all-K and Na-K media and NaCl was added to the all-Na media to equalize the total salt concentration of each medium.

All pH adjustments were made with a Beckman pH meter Model H2. A precaution was taken to avoid any misleading results due to a salt effect on the meter electrodes. It was observed that when the same amount of alkali was added to the Na-K media and the all-K media the pH of the all-K media was usually about .2 of a pH unit higher. Therefore, when the pH of each type of media was adjusted to the same indicated pH more alkali would be unintentionally added to the Na-K media.

To equalize the amount of hydroxyl ions in each medium, the amount of alkali required to give the all-K media its desired pH was first determined and then the same amount of alkali was added to the Na-K media.

Preparation of Media:

Again the general procedures described by Henderson and Snell were used (13). After the media were made up to one-half their final assay volumes, they were dispensed into 60 tube assay racks at 1 ml. per tube. The six tubes in each row received in addition 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 ml. respectively of solutions being used as growth-limiting standards. Water was added to make the total volume 2 ml. in each tube. Dispensing was accomplished by use of a Cannon Automatic Dispenser (14).

Sterilization and Incubation:

Due to the known fact that variations in the autoclaving period cause low reproducibility of assay results, a standard procedure for sterilization was followed. Before using, the autoclave was heated to approximately 121°C for several minutes. The pressure was released rapidly and the assay racks were inserted. The temperature was again brought to 121°C in as short a time as possible. The racks were sterilized at this temperature for exactly five minutes. Next the pressure was released slowly, over a period of one minute, to minimize any boiling. The racks were removed and allowed to cool to room temperature. Following inoculation as previously described the racks were incubated at 37±1°C for 60-72 hours.

Titration:

The amount of growth was determined by a titration of the acid produced in each tube. The titrations were accomplished with the aid of a quinhydrone electrode and a Cannon Automatic Titrator (14). The dispensing portion of the titrator was adjusted so that for every 100 counts recorded 4 ml. of approximately .05 N KOH were delivered.

It should be mentioned that, with respect to titration, all conditions which were to be critically compared were included in single experiments so that any variations in the galvonometer setting, strength of KOH, or ml. dispensed per titration counts, between assays, would not affect the results.

PART I

THE EFFECT OF pH ON THE POTASSIUM REQUIREMENT OF CERTAIN LACTIC ACID BACTERIA

(Part I of this thesis is essentially in the form of a paper which will be submitted to the Journal of Biological Chemistry for consideration for publication.)

The Effect of pH on the Potassium Requirement of Certain Lactic Acid Bacteria

By John N. Mills, Finn Wold and Robert J. Sirny

(From the Department of Agricultural Chemistry
Oklahoma Agricultural Experiment Station
Stillwater, Oklahoma)

Studies of the effects of the pH of the medium on the nutrition of lactic acid bacteria have been limited to a few reports, by workers who observed a reduced antagonism between certain amino acids as a result of a lower pH (1-4). MacLeod and Snell(5) stated that the potassium requirement was higher than one would expect for direct metabolic functions. These same workers later demonstrated that mineral antagonisms were responsible for a fluctuating potassium requirement.

In this report a pronounced effect of pH on the potassium requirement of certain lactic acid bacteria is presented.

Experimental

Cultures

Cultures of Lactobacillus arabionsus (ATCC 8014)[1], Streptococcus faecalis (ATCC 8043), Leuconostoc mesenteroides P-60

[1] American Type Culture Collection Number.

(ATCC 8042) and Lactobacillus delbrueckii (L. d. III Henneberg)[2] were carried as stabs in yeast glucose agar media and were transferred bi-monthly.

Basal medium

The basal medium used was essentially that recommended by Henderson and Snell (2) except for the substitution of equimolar amounts of soduim salts for the respective potassium salts. It should also be pointed out that ammonium chloride was not included in the medium because of the effect of the ammonium ion on the potassium requirement of lactic acid bacteria (5) and because of the related effect of pH on the solubility of ammonia.

Reagent grade chemicals were used and were dissolved in glass-distilled water. The potassium contamination, which was determined with the aid of a Perkin-Elmer Flame Photometer Model 52C, was 1×10^{-4} meq. potassium per tube. This amount of potassium present as a contaminant has been taken into consideration in all data presented in this paper.

The pH of the media was carefully adjusted by the addition of known amounts of standardized NaOH to aliquots of the complete medium with the aid of a Beckman Model H2 pH meter. Amounts of a standardized NaCl solution were then added to equalize the sodium content of each medium. Potassium was added quantitatively in the form of potassium chloride. All additions were made with a Cannon dispenser.[3]

Sterilization was by autoclaving for five minutes at 121°C.

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[2]Described by Sirny et al. in J. Nutrition 41, 383 (1950).
[3]International Instrument Company, Los Angeles, California

One drop of an inoculum prepared by resuspending cells in distilled water was added to each assay tube with a hypodermic needle. After approximately 65 hours of incubation at 37°C the acid produced in each tube was titrated with a Cannon titrator[4] and quinhydrone electrode assembly. Results are expressed as titration counts, with 100 counts approximately equal to 4 ml. of .05 N KOH.

This experiment was set up in triplicate in sixty tube racks with each tube containing 2 ml. of final-strength medium. The racks were autoclaved and titrated in three groups with one set of conditions in each group. Results are expressed as the average titration counts of three tubes.

Results

The design of the experiments in these studies consisted of a comparison of the amount of potassium required for one-half maximum growth at different pH levels. Ten different pH levels ranging from 4.3 to 8.0 were used. Maximum growth was considered to be that growth obtained when an excess (.05 meq./tube) of potassium was included in the medium for each pH studied.

The average titration count of the tubes containing no added potassium was subtracted from the average count of those in which growth occurred so that only a measure of the net acid produced was recorded. Due to the low buffer capacity of the citrate-buffered media at alkaline pH levels a correction for a negative blank was considered unnecessary; therefore, at

[4]International Instrument Company, Los Angeles, California.

alkaline pH levels the total titration count was recorded as a measure of the net acid produced.

Lactobacillus arabinosus

The amounts of potassium required by this organism for one-half maximum growth at various pH levels are shown in Figure 1. It is observed that 3.8×10^{-4} meq. potassium per tube are required at pH 6.5. At pH levels above or below 6.5 the requirement increases as the difference from pH 6.5 increases. At pH 8.0 the requirement is about twice as high as it is at pH 6.5, whereas at pH 4.3 the requirement is more than four times greater than it is at pH 6.5.

Leuconostoc mesenteroides

This organism has an optimum pH of 6.0 with respect to its potassium requirement. The requirement increases sharply as the pH is increased or decreased compared to pH 6.0. At pH 7.7 the requirement is nearly six times greater and at pH 4.3 it is nearly five times greater than its requirement of at pH 6.0. An increase in pH level from 6.0 to 6.5 doubles the requirement, as is the case for a decrease from the near optimum pH of 5.5 to pH 5.0.

Lactobacillus delbrueckii

Similar to the organism L. arabinosus, this organism has its minimal potassium requirement at pH 6.5. A slight increase in the requirement is seen at a pH 6.0. At all other pH levels, the requirement increases rather sharply as the difference between the pH studied and pH 6.0 is increased. At either pH

7.7 or 4.3 the requirement is about 7 times greater than that observed at pH 6.5.

Streptococcus faecalis

Unlike the other organisms studied, the pH optimum with respect to the potassium requirement for this organism was over a surprisingly broad range, with the requirement being at a constant and minimal value over the range pH 6.5 to 8.0. Another point of difference is the fact that the requirement does not increase with increasing alkalinity over the range studied. While the amount of potassium needed at pH levels above 8.0 was not determined, this would be of interest since, in related studies, maximal growth was obtained with initial pH levels as high as pH 10.0. A further observation meriting comment is that S. faecalis possesses a potassium requirement which is lower than the minimum required by any of the other three organisms studied. With pH levels below 6.5, the organism is seen to respond in a manner similar to the others studied.

While it is realized that absolute requirements are relatively meaningless unless considered as functions of the specific conditions employed, it is of interest to further compare the relative requirements of these four organisms. At any given pH there is a striking difference in the amount of potassium required. At pH 6.5 which is included in the optimal pH area for each organism the requirement is about 2.6×10^{-4} meq. per tube for S. faecalis which is somewhat less than the 3.8×10^{-4} meq. per tube required by L. arabinosus. The requirement at the same pH for L. delbrueckii is about 8.6×10^{-4} meq. per tube

Figures 1-4

The effect of pH on the potassium requirement for one-half maximal growth of certain lactic acid bacteria.

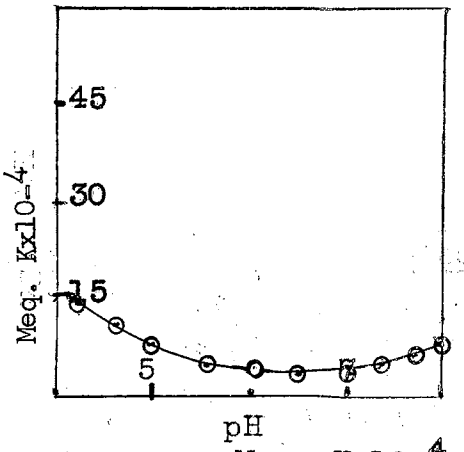

Figure 1. Meq. Kx10^{-4} required per tube by L. arabinosus.

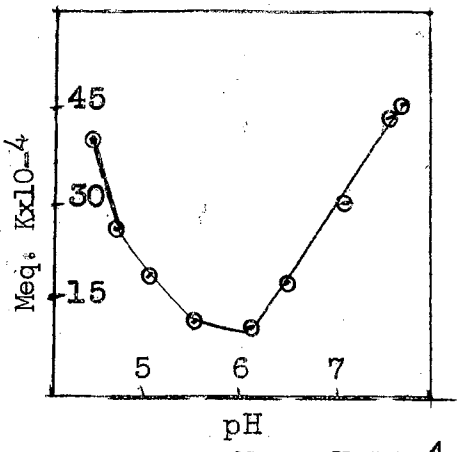

Figure 2. Meq. Kx10^{-4} required per tube by L. mesenteroides.

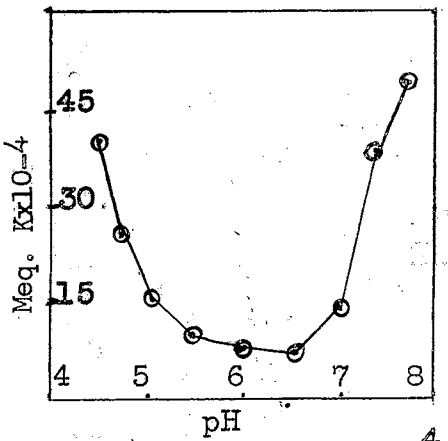

Figure 3. Meq. Kx10^{-4} required per tube by L. delbruckii 3.

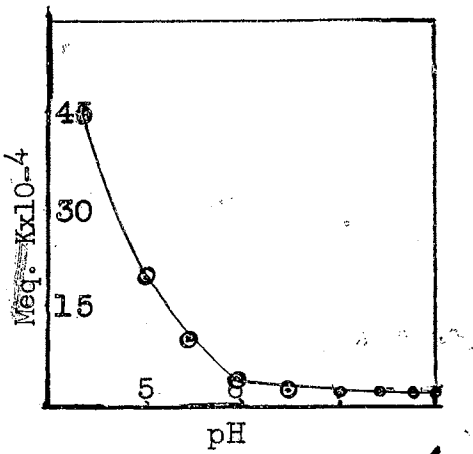

Figure 4. Meq. Kx10^{-4} required per tube by S. faecalis.

which is much less than the 16 x 10^{-4} meq. per tube required by L. mesenteroides.

Discussion

More information is needed regarding the effect of both the hydrogen ion concentration and the potassium ion concentration on the nutrition of lactic acid bacteria before the results presented here can be adequately explained. These results can only be compared in a very limited manner with other studies concerning the potassium requirement. Most other studies were usually conducted at the routinely used range 6.5-7.0. However, a recent report by MacLeod and Onofrey described some studies which were conducted at pH levels of 5.0 and 7.0. (6) While their studies were not designed specifically to determine the effect of pH requirement, their results included a comparison of the growth of L. arabinosus at these two different pH levels with various growth-limiting amounts of potassium. Relatively little difference in growth was obtained, and this was the basis for the author's suggestion that pH did not affect the response of the organism to potassium at these two pH levels. This observation, however, is not inconsistent with the findings in the studies being reported in this thesis, since the optimal pH of 6.5, at which the potassium requirement is minimal, was not used in their studies. Close inspection of their data reveals that there is actually less growth at pH 5.0 than at 7.0 with the same amount of limiting potassium. This is consistent with the results obtained here, and

it would be expected that a much greater difference would have been obtained if additional pH levels between 5.0 and 7.0 had been tested.

An approximation of the actual potassium requirement of these organisms was determined at pH 6.5 in some earlier work by MacLeod and Snell (5)(7), and the more specific requirements reported in this work fall in this rather wide range. The only noticeable difference is in the comparison of the requirements of the individual organisms which is not surprising in view of the different basal medium and technique used in the two laboratories.

A noteworthy observation in regard to the specific requirements reported here is that related studies have indicated that the optimum pH of these organisms is the same as the pH at which the least amount of potassium is required. In regard to the relatively low potassium requirement of S. faecalis it is of interest to note that MacLeod and Snell (7) have reported that the manganese requirement of this organism is also much less than that of certain other lactic acid bacteria.

While it is premature to form any conclusions regarding the metabolic functions of potassium which are regulated in some manner by pH, the importance of pH on mineral metabolism is obvious. Further studies will undoubtedly reveal more very striking relationships between pH and the nutritional requirements of lactic acid bacteria.

Summary

The response of four lactic acid bacteria to graded amounts of potassium at ten different pH levels was studied. A striking effect of pH on the potassium requirement of each organism was noted. Variations with regard to the amount of potassium required for each organism at specific pH levels were noted.

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PART II

STUDIES ON A POSSIBLE SODIUM REQUIREMENT IN LACTIC ACID BACTERIA

A. Specific Conditions Altering Certain Mineral Requirements of Lactic Acid Bacteria.

The importance of an evaluation of the conditions altering mineral requirements in a study of mineral nutrition was implied by MacLeod and Snell (5) who said,

> "The fact that a given inorganic ion is not essential for growth under a given set of conditions does not discount the possibility that it may be useful in metabolism or that it may even become essential under another set of conditions."

These workers conducted rather extensive studies of some interrelationships between certain minerals in the nutrition of lactic acid bacteria and have reported their results in a series of publications(4)(5)(15). The studies reported in this thesis have further elucidated certain of these interrelationships, especially those involving potassium, sodium and ammonium. These relationships were found to depend not only on the concentrations of the involved cations but also on the concentrations of certain interrelated amino acids and especially on the pH of the medium. Differences between specific organisms with respect to the effects of these factors were also noted.

The following section is devoted to a consideration of

typical illustrations of each of these conditions which affect the mineral requirements. These conditions will be considered for each of the illustrations with a cognizance that the effects of one factor cannot be isolated from the effects of others.

Effects of Other Minerals:

The effects of related ions on the potassium requirement of lactic acid bacteria have been extensively investigated by MacLeod and Snell (15). It was of interest to determine the effect of certain related ions on an assumed sodium requirement of these organisms. This assumption includes within it the supposition that the sodium requirement is met by sodium contamination of the nutrients present. If this assumption is true, near-maximum growth would be expected with those conditions set forth in the General Procedures, and this is the case.

It should be possible to demonstrate a sodium requirement if the requirement could be increased in some manner. This suggests the use of a method analagous to that of increasing the potassium requirement by increasing the concentration of its antagonists, namely ammonium and sodium ions. The similarity of the chemical nature of sodium and potassium favors the assumption that the ammonium ion would also antagonize the sodium ion. The results of adding 6 mg. per tube of ammonium chloride to a medium supporting the growth of L. Arabinosus at a pH 8.0 are shown in Figure 1.

This figure illustrates the response of the organism to limiting amounts of valine at an initial pH of 8.0. It is

observed that very substantial growth was obtained with the all-K and the Na-K media which did not contain ammonia. Essentially equal growth was obtained when ammonium chloride was added to the Na-K medium, but in the all-K medium there was a relative inhibition of growth by the ammonium ion.

This may be interpreted as an indication that the ammonium ion increases the sodium requirement to such an extent that the requirement is not met by contamination. The relatively high amounts of sodium and potassium in the Na-K medium offset any antagonistic effect of ammonia on the requirement of either sodium or potassium.

In these studies it has also been observed that there is an effect of the ratio of sodium and potassium on the response of an organism to a limiting nutrient in the presence of different amounts of salts C (Appendix B). This is illustrated in Figure 2. It is observed here that the response of L. mesenteroides to limiting amounts of glycine varies with the concentration of sodium and potassium in the media containing different levels of salts C.

It is not possible to explain these results on the basis of present information; however, it should be noted that sodium and potassium are in some manner involved in certain favorable and unfavorable interrelationships with one or more of the constituents of salts C.

Effects of Amino Acids and Vitamin Concentrations:

Impressed with MacLeod and Snell's (15) finding that sodium and ammonium ions were antagonistic with respect to potassium,

Sirny (16) investigated the effect of various ratios of sodium and potassium in media normally used in microbiological assays. Sirny and coworkers (17) (18) found that sodium in the media decreased the response of an organism to a limiting nutrient in most cases studied. This was especially true if the ratio of sodium to potassium was high, as is the case in the commonly used Henderson-Snell medium (13). The most striking exception was noted in the response of L. arabinosus to limiting amounts of pantothenic acid. In this case the omission of sodium from the medium was determintal to the growth of the organism.

In the studies reported here similar effects of sodium on the response of an organism to a limiting amino acid were noted, as illustrated in Figure 3. In the study of this striking amino acid interrelationship, first reported by Sirny et al (19), it is observed that there is a detrimental effect on the growth if the Na-K medium is substituted for the all-K medium. These studies also revealed conditions other than pantothenic acid utilization under which high sodium in the medium stimulated growth.

Certain factors affecting the ability of L. mesenteroides to synthesize serine in the presence of adequate amounts of glycine have been extensively investigated by Wold (20). In an investigation of the sodium and potassium effect on serine synthesis it was found that sodium also affects serine synthesis. Some results of these studies are shown in Figure 4, and a definite stimulation of growth is seen when the Na-K medium is compared to the all-K medium.

In a subsequent experiment these two conditions under

which a sodium stimulation is obtained were combined to determine if sodium was even more beneficial in promoting serine synthesis under the stress of limiting pantothenic acid. The results are shown in Figure 5. Although a striking sodium stimulation is shown it is not believed to be significantly greater than that shown either for pantothenic acid utilization or serine synthesis under conditions used in earlier experiments.

A further stimulatory effect is illustrated in Figure 6. It is observed here that sodium favorably affects the synthesis of another amino acid, threonine. While not directly pertinent to this observation, it is desirable to note that from other studies conducted that serine and threonine themselves do not appear to be interrelated in the metabolism of this organism.

Thus, it is seen that sodium favorably affects the synthesis of serine and threonine. However, the precise meaning of this observation is again not apparent. It is possible that sodium is involved in the activation of certain enzyme systems which function in the synthesis of these amino acids, or sodium may be involved in the deactivation of enzyme systems which are antagonistic to their synthesis. The same may be true concerning the enzyme systems which involve pantothenic acid utilization.

Effects of pH and Related Factors:

The striking effect of pH on the potassium requirement of certain lactic acid bacteria (Part I) suggests a similar effect of pH on an assumed sodium requirement. A study of the

effects of the pH of the medium on the growth of these organisms is invariably also a study of the effects of various concentrations of carbon dioxide and ammonia on their growth. The concentrations of these gases is further regulated by the incubation temperature. The following discussion will particularly deal with the effects of pH and ammonia as related to a possible sodium requirement.

It was of interest to determine the effect of ammonium ions at different hydrogen ion concentrations with respect to the all-K and Na-K media. It was found with the all-K medium, as shown in Figure 7, that ammonium ions present in thrice the concentration recommended by Henderson and Snell (13) were toxic at pH 6.0 but stimulatory at a pH of 7.5. The same stimulatory effect is observed with the Na-K medium at a pH of 7.5, but at a pH of 6.0 the ammonium ions have no significant effect. The non-stimulatory effect of ammonium ions at this latter pH may actually be due to mineral antagonism as a result of the high amount of ammonium ions retained in the medium at an acid pH as compared to the relatively small amount retained at an alkaline pH. Quantitative determination of the amount of ammonia lost at the alkaline pH was not made, but this is a definite possibility for further evaluation of these ammonia effects. In the case of the all-K medium (at pH 6.0), with which an actual inhibition is observed by ammonia, it is possible that the inhibition is due to an increase in the sodium requirement caused by the high amount of ammonia present. Should this be the case, a similar inhibition would not be expected with the Na-K medium.

Related effects of pH on a possible sodium requirement will be further dealt with in the following section.

Figures 1-2

The effect of certain other minerals on the response of two organisms to the Na-K and all-K media.

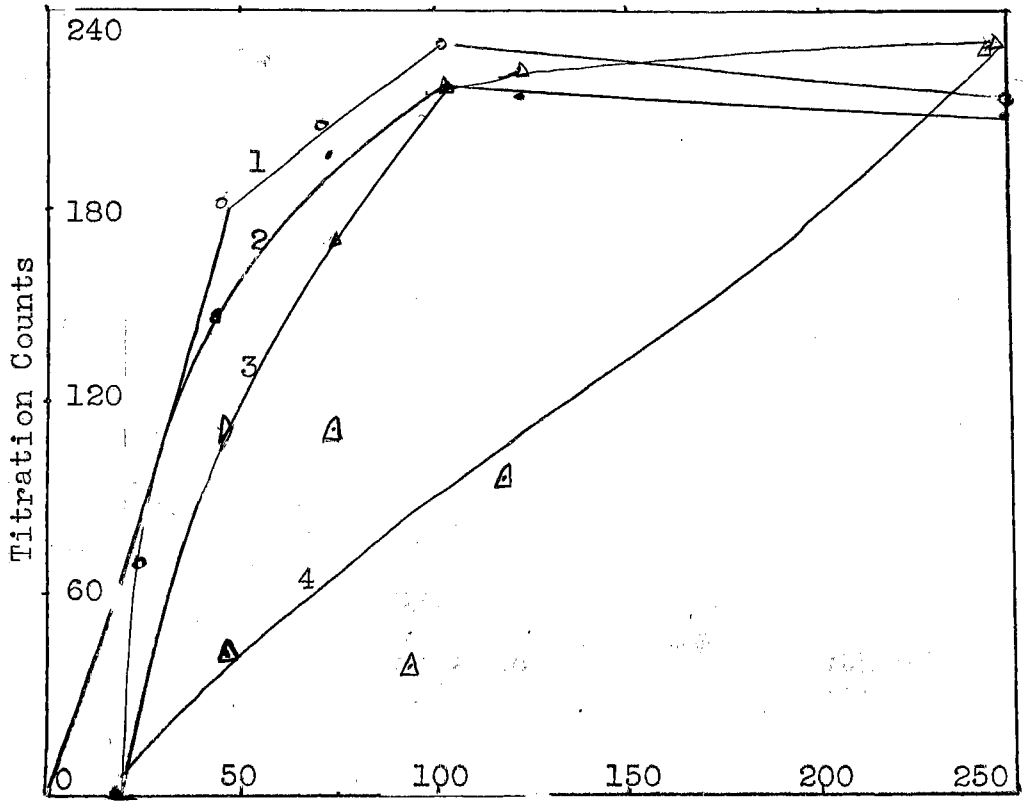

Figure 1. Effect of ammonium ion on growth of L. arabinosus. (1) Na-K, (2) Na-K-NH_4, (3) all-K, (4) all-K-NH_4.

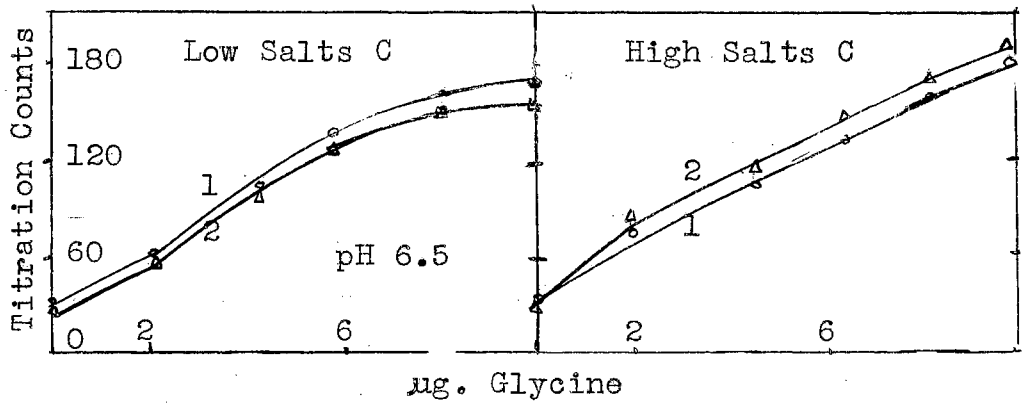

Figure 2. Effect of the amount of salts C (Mn, Mg, Fe) on the growth of L. mesenteroides. (1) all-K, (2) Na-K.

Figures 3-4

Effect of the Na-K and all-K media on the response of L. mesenteroides to certain aspects of amino acid metabolism.

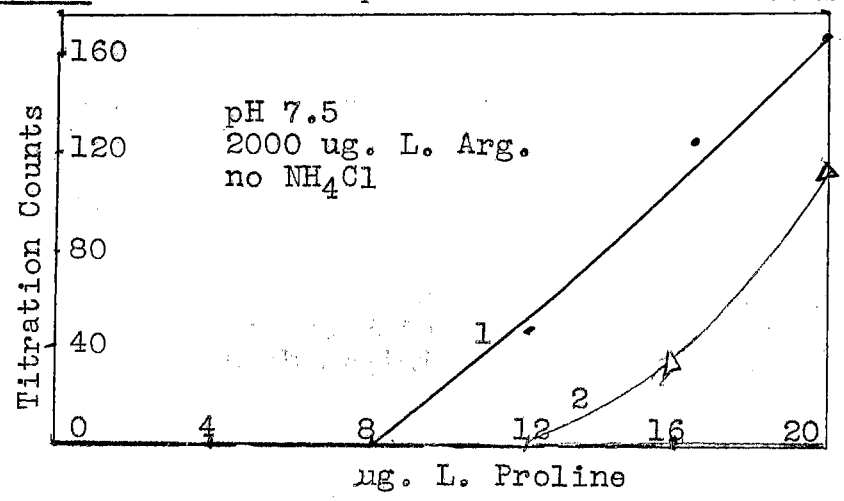

Figure 3. Effect on proline utilization in an amino acid interrelationship requiring high arginine. (1) all-K, (2) Na-K.

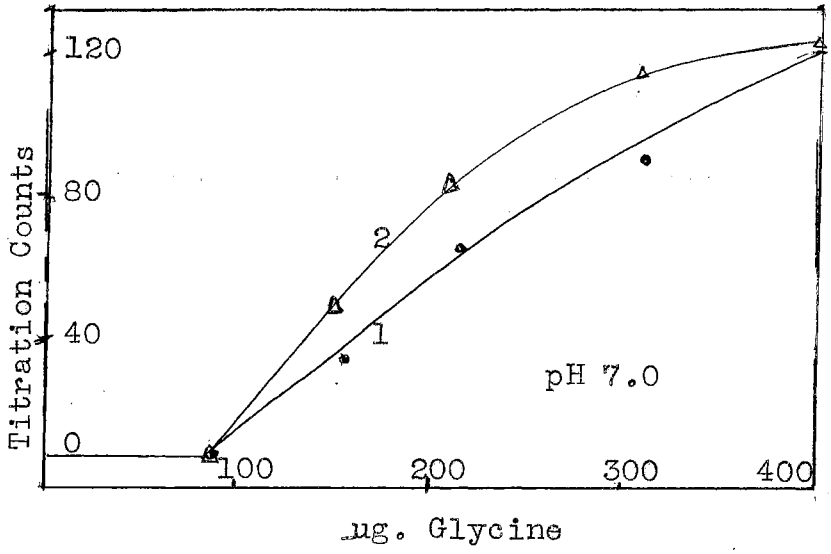

Figure 4. Effect on serine synthesis. (1) all-K, (2) Na-K.

Figures 5-6

The effect of Na-K and all-K media on response of L. arabinosus to limiting Pantothenic acid under stress of amino acid synthesis.

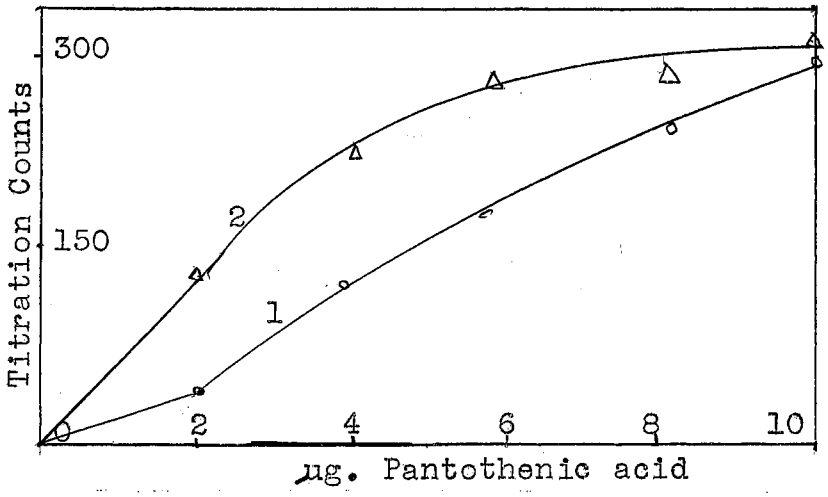

Figure 5. Serine Synthesis. (1) all-K, (2) Na-K.

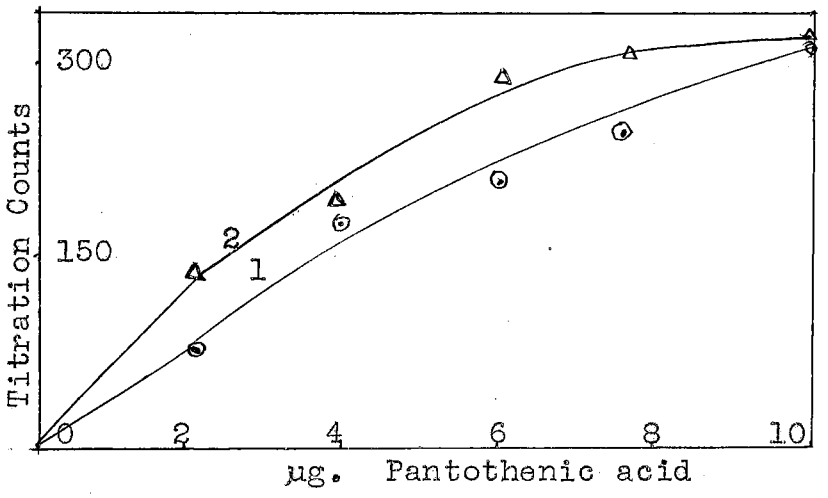

Figure 6. Threonine Synthesis. (1) all-K, (2) Na-K.

Figure 7

Variation of ammonium effect with pH in an all-K medium containing 50 ug. Arg.-HCl on the response of L. mesenteroides to limiting proline.

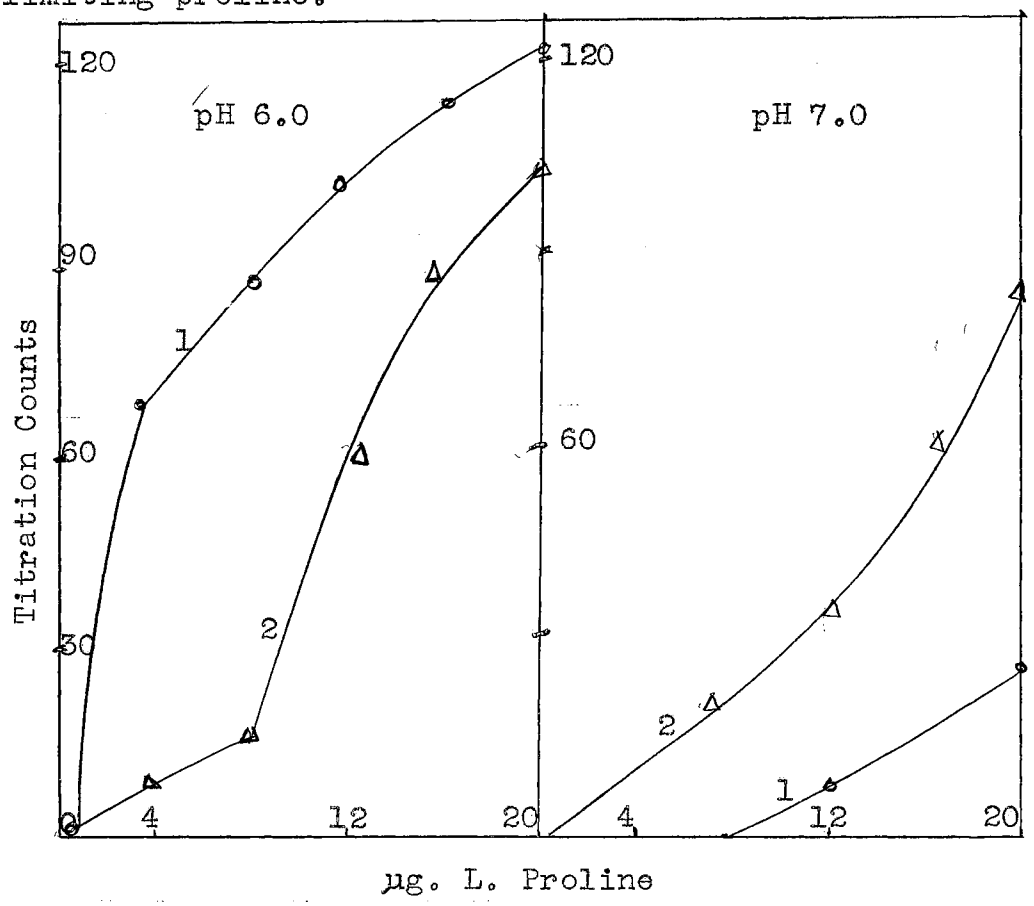

Figure 7. (1) no ammonium, (2) with ammonium.

B. Specific Conditions Indicating a Sodium Requirement of Lactic Acid Bacteria.

A requirement or biochemical role for sodium has never been demonstrated in lactic acid bacteria. The demonstration of a requirement of a mineral in these organisms is essentially a problem of obtaining a medium which contains an insufficient amount of the trace mineral for supporting maximum growth. This problem may theoretically be solved by reducing the amount of the trace mineral as a contaminant or by varying the conditions under which the organism grows so that the requirement is increased. The latter method has previously been mentioned in regard to mineral antagonisms.

Attempts to demonstrate a sodium requirement by using a more highly purified medium were not successful. Maximum growth was supported by media containing only reagent grade chemicals dissolved in glass-distilled water. The sodium contamination in the carefully prepared medium was about 7 parts per million as determined with the aid of a Perkin-Elmer Flame Photometer Model 52C.

An attempt was made to further purify the medium by "pre-treatment" of the medium with some organism which possesses a specific requirement for the trace mineral. This method essentially involves growing the organism in double-strength media, removing these organisms, readjusting the pH and reinoculating the media with the test organism. This method was tried, but no suitable organism was found which removed sufficient sodium from the medium. MacLeod (21) has reported an apparent sodium

requirement of a marine bacterium, but further investigation showed that the sodium was not found inside the cell wall.

In view of the striking effect of pH on the potassium requirement (Part I) it seemed very probably that in addition to mineral antagonism a change in the pH of the media might affect a sodium requirement, if it existed. The effect of pH on the growth of L. arabinosus in the all-K and Na-K media is shown in Figure 7. It is seen here that at pH 8.3 there is a striking difference in the response of this organism to the two different media. This is interpreted as meaning that at this pH the sodium requirement is not satisfied by the sodium present as a contaminant in the all-K medium. Despite this indication of a sodium requirement the fact that sodium in the Na-K medium is present in a relatively high amount must be considered. When a salt stimulates growth only at high levels its effect is probably not specific. Thus, it was desirable to demonstrate a sodium requirement in a more positive manner.

The best evidence obtained thus far to indicate a sodium requirement is illustrated in Figure 8. These results were obtained from an experiment designed to study the response of the organism L. arabinosus to limiting amounts of sodium ions in an all-K medium at an initial pH of 8.2. Except for the omission of ammonium chloride this medium contained the concentrations of all the nutrients described in Appendix B. Therefore, the effective factor appears to be the high pH, which increases the sodium requirement in a manner similar to its effect on the potassium requirement in this same organism, as has been presented in Part I.

It should be particularly noted that no growth, as evidenced by the low blanks, was obtained in the absence of sodium, and that the growth response obtained to the added sodium is promoted by definitely trace amounts of sodium. Thus maximum growth is obtained with a level of sodium as low as 0.0008 milliequivalent per tube, and it may be pointed out further that this maximum growth is excellent growth on this medium and at this pH.

This direct evidence of a sodium requirement in L. arabinosus under these conditions appears to be, in itself, a good indication that sodium plays an important role in the metabolism of this organism. This is further supported when viewed from the standpoint of other previously mentioned indications of a sodium requirement, such as the favorable effect of sodium on pantothenic acid utilization, serine and theonine synthesis, and the relationship of sodium to the effect of ammonia in the medium. In view of all these observations, it is believed that the role of sodium in this organism, and possibly in many others, will be found to be a relatively specific one, and that the order of magnitude of the requirement will be similar to that for potassium.

Figure 8-9

The effect of pH on a sodium requirement in L. arabinosus

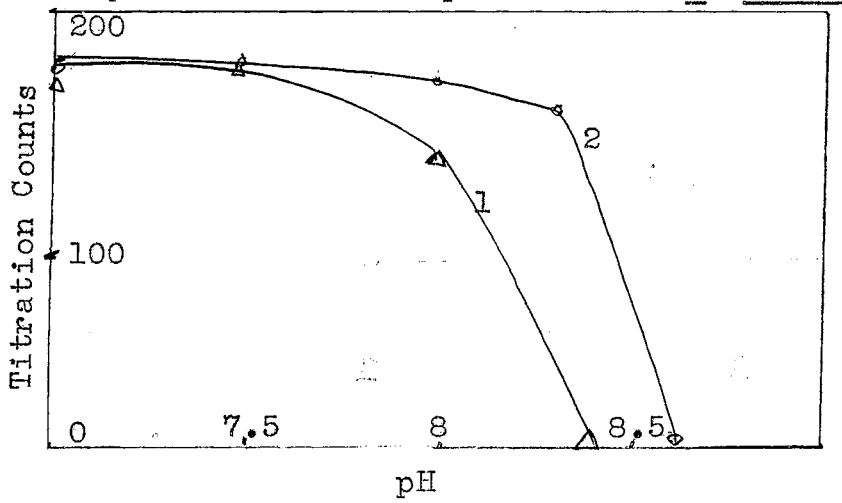

Figure 8. Response to all-K and Na-K at alkaline pH levels. (1) all-K, (2) Na-K.

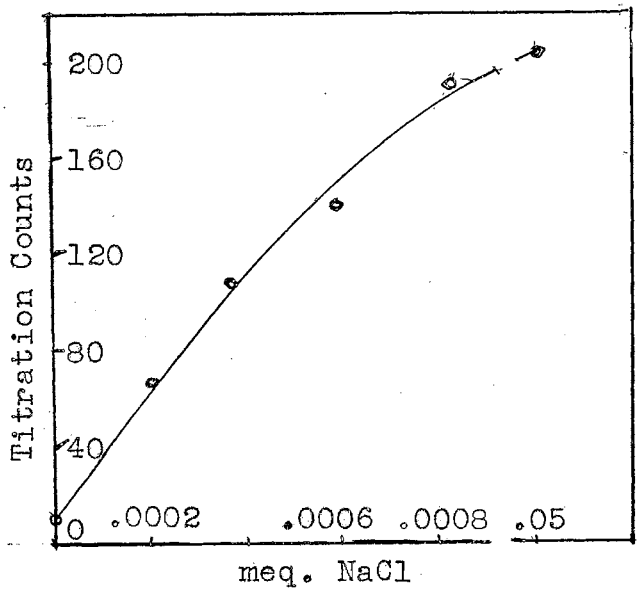

Figure 9. Response to limiting amounts of sodium at pH 8.2.

GENERAL DISCUSSION

The importance of knowledge pertaining to the mineral nutrition of lactic acid bacteria has been made more apparent by these studies which elucidate further the role of minerals as part of a delicate balance of numerous interrelated factors. The chief regulator of this delicate balance appears to be the hydrogen ion concentration of the medium. Very few studies have been conducted concerning the effect of pH on the metabolism of these organisms. The work reported here is a very brief introduction to the relationship between mineral nutrition and pH in certain related metabolic studies.

Indications that essentially the same factors which affect the requirements of one mineral also affect an assumed requirement of another encourages a closer evaluation of all factors affecting known mineral requirements. These investigations were especially concerned with a parallel study of the factors affecting a known potassium requirement with those affecting an assumed sodium requirement. A potassium requirement has been established (15) but the study of factors affecting its requirement have been limited to those involving certain related mineral ions.

The studies reported in this thesis have demonstrated a striking pH effect on the potassium requirement of certain lactic acid bacteria. In a consideration of the pH effect on

any requirement in these organisms, an evaluation of the known significance of the initial pH of the medium is very necessary. These organisms have pH optima which are surpassed during their growth as a result of acid production. The assay media is therefore buffered so that sufficient growth may occur to permit a practical study. The buffer capacity of the citrate-buffered medium used in these studies is greatest from pH 7.0 to 5.0. This means that growth beyond this range is limited. The growth produced from the use of a medium at any pH is only a summation of growth occurring while the pH changes from the initial pH to a toxic pH unless the supply of some nutrient is depleted before this toxic pH is reached. If the initial pH is above 5.0 most of the growth will occur somewhere between pH 7 and 5.

Another factor which must be considered is that of the relationship between sterilization and the initial pH of the medium. It is known that autoclaving causes chemical charges to occur in the medium. Certain amino acids are destroyed (17) during autoclaving, a color change attributable to carmelization of the glucose occurs, and certain amino acids are found in less available forms with the glucose of the medium. Certain beneficial changes are also known to occur, and it has been suggested that under certain conditions, stimulatory growth factors are formed for some organisms. It is expected that many of these factors would be affected by pH. Furthermore, the pH of a medium may be changed considerably as a result of new products formed and gases as ammonia and carbon dioxide given

off. Thus the effect of the initial pH of the medium on the response of an organism is influenced by the effect of sterilization at this pH.

Further effects of pH are concerned with its effect on the amount of carbon dioxide and ammonium retained in the medium. These latter factors are also regulated by the incubation temperature. Studies involving carbon dioxide tension and temperature effects have been limited chiefly to studies involving the ability of L. arabinosus to synthesize phenylalanine and tyrosine. Lyman et al. (10) showed that this organism could synthesize these amino acids if the basal medium was supplied with a 6 per cent carbon dioxide gas phase over the liquid culture. Borek and Maelsh reported that a temperature increase of 2° (from 35° to 37°) changed the status of phenylalanine from that of a non-essential nutrient to an essential nutrient. The nutritional requirements of several mutants have been shown to depend upon the pH or the temperature (12). As all of these mutants have more exacting nutritional requirements at lower pH levels or at higher incubation temperatures it appears that the mutants' ability to utilize carbon dioxide, in the synthesis of essential nutrients, is involved.

A consideration of pH effects on the nutritional requirements of these organisms would be incomplete without reference to the pH optima of the many enzyme systems involved in the cellular metabolism. The summation of these pH optima

determine a loosely defined pH optimum of the specific organism while growing under specific conditions.

The most important function of trace minerals has been attributed to their catalytic function in enzyme systems. Various mechanisms by which minerals function in enzyme systems have been suggested. One involves the oxidation or reduction of the metal as a result of its ability to accept or donate electrons to cause a substrate to be respectively reduced or oxidized. Another mechanism involves activation of otherwise inactive enzyme systems by the mineral ions. The exact nature of this mechanism is not understood, but according to some theories the metal activates by entering into a chelated type of combination between the protein part of the enzyme and its substrate. Thus the varying degrees of specificity for metals exhibited by different enzymes could be explained on this basis, and this in turn, would explain the sparing effect of some minerals on the requirements of others.

A striking effect of the initial pH of the medium on the mineral requirement seems only as would be expected in view of the complicated factors affected by the pH of the medium. These complicated factors make it extremely difficult, in view of the limited knowledge pertaining to mineral metabolism, to identify which factor or group of factors are affected in such a way as to change the requirement for the mineral involved. The studies reported in this thesis indicate a number of instances in which sodium favorably affects the growth of the organism. These seemingly uncorrelated

instances have been observed many times in repeated experimentation. The number of instances, these repetitions and their reproducibility strengthens the possibility of the elucidation of specific roles of sodium in these organisms. Furthermore, the fact that the conditions favoring a sodium stimulation do not appear to be correlated is not disturbing in view of the limited information available concerning the complex interrelationships involved.

The demonstration of a sodium requirement at a particular pH indicates that the network of interrelationships at this pH involves a combination of conditions, some of which have been reported here, under which sodium is favorably involved to a higher degree.

Suggestions of a general mechanism which would be compatible with the observations reported in this thesis is difficult. However an attractive possibility may be that the permeability of the cell wall to various nutrients is the fundamental area involved in these complex interrelationships between pH, inorganic ions and organic nutrients. Some advances are presently being made in this area, and it will be of real interest to learn whether or not herein will be found the explanation of these complex interrelationships.

GENERAL SUMMARY

In these studies, investigations of the sodium and potassium requirements of lactic acid bacteria have revealed a number of conditions which affect both of these mineral requirements. Extensive studies were made of certain conditions which affect the potassium requirement. These conditions included certain amino acid concentrations, the presence of certain mineral antagonists and especially the pH of the medium. Knowledge of these factors was then applied to studies designed to demonstrate a sodium requirement. It was found that sodium favorably affects pantothenic acid utilization, serine and threonine synthesis, growth in media containing ammonium salts, growth in media containing a high concentration of manganese, magnesium and ferrous ions, and especially growth in alkaline media. The demonstration of a striking pH effect on the potassium requirement was followed by attempts to demonstrate a sodium requirement at alkaline pH levels.

Using the organism Lactobacillus arabinosus excellent response to trace amounts ($< .001$ meq./tube) of sodium was obtained in otherwise complete medium at an initial pH of 8.2. This has been interpreted as evidence of a sodium requirement in this organism.

Possible explanations are presented to account for the effect of pH on the potassium and sodium requirements of lactic acid bacteria.

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APPENDIX

APPENDIX

A. Media for Storage and Transfer of Organisms.

Agar medium:

Yeast extract	10.0 gm.
Glucose	2.5 gm.
Agar	15.0 gm.
K-acetate	5.0 gm.
Water to 1000 ml.	

Liquid transfer medium:

Glucose	1.0%
K-citrate	1.0%
K-acetate	0.1%
K_2HPO_4	0.5%
NH_4Cl	0.3%
Tryptone	0.5%
Yeast extract	0.5%
Salts C soln.*	1.0%
Vitamin soln.*	0.5%

Dissolved in water, and pH adjusted at 6.0

The media were sterilized and stored in the refrigerator.

B. Basal Media for Microbiological Assays

Amino acid mix**(for 100 tubes at 2 ml. final assay volume):

DL-Alanine	200 mg.	DL-Threonine	40 mg.
DL-Aspartic acid	200 mg.	DL-Tryptophan	40 mg.
L-Glutamic acid	200 mg.	DL-Valine	40 mg.
L-Arginine.HCl	40 mg.	Glycine	20 mg.
DL-Isoleucine	40 mg.	L-Cystine	20 mg.
L-Lysine.HCl	40 mg.	L-Histidine.HCl	40 mg.
DL-Methionine	40 mg.	L-Leucine	20 mg.
DL-Phenylalanine	40 mg.	L-Proline	20 mg.
DL-Serine	40 mg.	L-Tyrosine	20 mg.
		Made up to 50 ml. with acid and heat.	

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* Composition given in Appendix B.
**The amino acid assayed for, to be omitted.

B. (Continued)

Sugar mix (for 100 tubes at 2 ml. final assay volume):

Glucose	4.0 gm.
K-citrate.H_2O	4.4 gm.
K-acetate (anhydr.)	0.2 gm.
NH_4Cl	0.6 gm.
K_2HPO_4	1.0 gm.
Salts C soln.	4.0 ml.
AGU-soln.	2.0 ml.
X-soln.	2.0 ml.
Vitamin soln.	2.0 ml.

50 ml. of amino acid mix is added, and the total made up to 100 ml. pH adjusted to the desired value.

Solutions for the above sugar mix:

Salts C

$FeSO_4.7H_2O$	0.5 gm.
$MnSO_4.7H_2O$	2.0 gm.
$MgSO_4.7H_2O$	10.0 gm.

Dissolved with the aid of HCl, and made up to 250 ml.

AGU-soln.

Adenine-sulphate	250 mg.
Guanine, HCl	250 mg.
Uracil	250 mg.

Dissolved with the aid of HCl and made up to 250 ml.

Vitamin soln.

Thiamin	25.0 mg.
Niacin	25.0 mg.
Ca-pantothenate	25.0 mg.
Pyridoxal	5.0 mg.
Riboflavin	25.0 mg.
PABA	5.0 mg.
Biotin*	0.25 mg.
Folic acid**	0.25 mg.

Riboflavin dissolved first with hot water and acid, then the rest of the vitamins added and volume made up to 250 ml.

X-soln.

Xanthine	250 mg.

Dissolved in dilute KOH and made up to 250 ml.

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* Biotin stored in soln. in 50% EtOH.

**Folic acid stored in soln. in dil. KOH or NaOH in 50% EtOH.

VITA

John Norman Mills

candidate for the degree of

Master of Science

Thesis: INVESTIGATIONS OF THE SODIUM AND POTASSIUM REQUIREMENTS OF LACTIC ACID BACTERIA.

Major: Chemistry

Biographical and Other Items:

Personal data: Born at Neenah, Wisconsin, September 29, 1932, the son of Norman J. and Ruth M. Mills.

Education:

Undergraduate Study:

1950-1954 Wisconsin State College - Oshkosh

Graduate Study

1954-1956 Oklahoma A. and M. College

Professional experiences:

1952 (summer) Stokleys Canning Company

1953-1954 Oshkosh Water and Softener Company

1953 (summer) United States Rubber Company Arsenal at Joliet, Illinois.

Member of American Chemical Society, Phi Lamda Upsilon, and Phi Sigma.