INVESTIGATION OF THE BIOSYNTHESIS OF GLYCINE IN LACTOBACILLUS BREVIS

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INVESTIGATION OF THE BIOSYNTHESIS OF GLYCINE IN LACTOBACILLUS BREVIS

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PREFACE

The knowledge that has been obtained by the use of the microbiological assay technique and from the study of amino acid, vitamin and mineral interrelationships in bacteria has had essential application to higher forms of life.

Microbiological studies have contributed much to our fundamental knowledge of amino acid biochemistry, and it has become recognized that many other factors, some of which were mentioned above, are intimately involved in the metabolism of the amino acids. Accordingly application of microbiological technique offered an improved opportunity to study experimentally certain stimulatory and inhibitory factors associated with the biosynthesis of glycine.

The author wishes to express his sincere appreciation to Dr. Robert J. Sirny, Associate Professor of Agricultural Chemistry Research, under whose direction this study was conducted, for his supervision and guidance throughout the course of the graduate work.

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CHAPTER I

INTRODUCTION

The biochemistry of lactic acid bacteria is very complex and varied from the standpoint of their cellular metabolism as well as from the standpoint of interrelationships between required nutrients. Some information regarding the differences between the species in this group is found in the bacteriological classification of these microorganisms. It is considered desirable to present a general description as well as certain aspects of this classification, as follows:

Lactic acid bacteria: Cocci and rods occurring singly, in pairs and in chains, ferment carbohydrates readily with production of lactic acid and some volatile acid. Like all other living organisms, lactic acid bacteria require for growth mineral salts, and a utilizable source of energy and nitrogen. They also requie an assortment of growth factors or vitamins. For their energy supply lactic acid bacteria are dependent upon the presence of carbohydraies.

The family of Latobacteriaceae fall into two large tribes, five main genera and 57 species. (1)

The Tribes of Family Lactobacteriaceae

I. Cocci occurring in pairs and chains.

Tribe I: Streptococceae

II. Rods occurring singly, in pairs and in chains.

Tribe II: Lactobacilleae

The Genera of the Tribe Streptococceae

1. Diplococcus: Cells in pair, gram positive, aerobic species bile soluble, 7 species
2. Streptococcus:Cells in chain, not soluble in bile, 21 species
3. Leuconostoc: Cells normally occurring as spheres. Gram positive, by-products of the fermentation of carbohydrates yield CO_2, acetic acid, lactic acid and ethanol, 3 species.

The Genera of The Tribe Lactobacilleae

1. Lactobacillus: Always produce lactic acid from carbohydrate, catalase negative, 15 species.
2. Propionibacterium: Ferment carbohydrates, polyalcohols and lactic acid with the formation of propionic, acetic acid and CO_2. Catalase positive, 11 species.

Knowledge of the composition and requirement of the lactic acid bacteria has been constantly expanding during the last quarter of a century. It has become apparent that not only the use of the microbiological assay technique and the findings from studies of the amino acid, vitamin and mineral interrelationships but also many general aspects of the cellular metabolism have essential application to higher forms of life as well as to microorganisms.

In recent years the industrial application of the lactic acid bacteria has been extended further, with the discovery that the organisms can convert sucrose into high yields of effective plasma substitutes. It has also been found that some of the lactic acid bacteria produce

antibiotic substances (2, 3, 4, 5).

Microbiological Techniques

Based on our increasing knowledge of the nutrition of the lactic acid bacteria, the microbiological assay technique for vitamins (6-12) and amino acids (13-23, 25, 26, 27, 34-37) has developed rapidly during the last fifteen years. The practical application of such assay was first reported by Snell and Strong (24) in 1939, and they are now among the most important analytical methods in protein and vitamin chemistry. (24, 25, 26) Pertinent review on the metabolism and nutrition of lactic acid bacteria has been published by Snell (28, 29, 30), Peterson et al. (31), Wood et al.(32) and Tittsler et al. (33).

In developing microbiological methods for the determination of amino acids, vitamins and other substances of nutritional importance, it is desirable first of all to have as complete detailed information concerning nutritional requirements of the organism as is possible to obtain. These procedures for determining amino acids have been worked out in a number of different laboratories. It has become apparent that amino acid requirements of the lactic acid bacteria are not always fixed; differences in the composition of the medium and in the conditions of the test can result in qualitative as well as quantitative differences in the amino acid requirement of some of these organisms.

This problem is exemplified by the synthesis of threonine in the presence of pyridoxal only by Lactobacillus arabinosus 17-5 (39) and the synthesis of aspartic acid in the presence of uracil by Lactobacillus casei (40).

The vitamin requirements of numerous lactic acid bacteria have also been investigated by many workers (9, 41, 39, 42, 25). Apparently, vitamin requirements are also altered by the presence or absence of other nutrients in the medium, as is exemplified by the replacement by alanine of the pyridoxal requirement of Streptococcus faecalis R (38).

Thus, it is seen that the nutritional requirements of bacteria are not only dependent on the vitamin levels in the medium but also on the levels of the amino acids.

Amino Acid Interrelationships

There is abundant evidence of amino acid interrelationships in regard to amino acid metabolism (43, 44, 45, 46). In recent years there have also been a number of reports that peptides exhibit greater growth promoting activity than free amino acid for microorganisms (47, 43, 45, 46).

An unusual interrelationship between arginine and proline in Leuconostoc mesenteroides P-60 has been reported by Sirny et al. (43) in which relatively high amounts of arginine are required for utilization of growth limiting amounts of proline. An effect of minerals on another type of amino acid interrelationship is that reported by Sirny and Mills (49) in which change in the relative sodium-potassium concentrations were found to markedly alter the degree of inhibition of valine utilization by either leucine or isoleucine in Lactobacillus arabinosus. It was also pointed out in these two latter reports that the utilization of peptide sources of the amino acids was inhibited to a lesser extent than was that of free amino acids. These interrelationships will be discussed further with respect to interrelationships involving alanine,

glycine and serine.

The Metabolic Interconversion of Serine and Glycine

The metabolic interconversion of serine and glycine was demonstrated first in the intact rat (50, 46, 51). That a similar metabolic relationship between serine and glycine also exists in an auxotrope (a mutant strain requiring for growth an exogenous source of a specific amino acid) of Escherichia coli which can grow in media containing either serine or glycine has also been reported (52, 53, 54). Recently, Abelson (55) has reported that on the basis of experiments with an "isotopic competition" technique Escherichia coli strain B can form glycine from serine but does not carry out the reverse reaction.

An up-to date study of the enzymatic conversion of serine to glycine and the reverse process has been shown to occur in a variety of bacteria (56, 57). In almost all instances, sensitive tracer technique, with C^{14} or N^{15} labeled substrate, have made it possible to study the reaction even though the net conversions were small.

The mechanism by which glycine is synthesized by Lactobacilli is still unknown. The present investigation was designed to reevaluate the amino acid interrelationships involved and to study the other factors which affect the biosynthesis of glycine in Lactobacillus brevis.

CHAPTER II

GENERAL PROCEDURE

Assay Cultures

Lactic acid bacteria are dependent on various carbohydrates for their principal energy source. Their action upon glucose has been used to classify these organisms into two large groups, homofermentative and heterofermentative.

The homofermentative group converts the glucose almost quantitatively to lactic acid (Glucose $\longrightarrow$ 2 lactic acid), and included in this group are the following:

Lactobacillus casei (ATCC 7469)

Lactobacillus delbrueckii (L.D III. Henneberg)

Lactobacillus arabinosus 17-5 (ATCC 8014)

Lactobacillus leichmannii (ATCC 7830)

Streptococcus faecalis R (ATCC 8043)

The heterofermentative group which also produces lactic acid from glucose produces, in addition, ethanol, CO_2 and acetic acid. The optimum pH for growth of most of these organisms lies between 6 and 7.

Leuconostoc mesenteroides P-60 (ATCC 8042)[1]

Lactobacillus brevis (ATCC 8287)

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[1] Identified as Pediococcus cerevisiae by Felton, et al., J. Bact. 65, 482, (1952)

Lactobacillus lycopersici (ATCC 4005)[1]

Leuconostoc citrovorum (ATCC 8021)

Lactobacillus brevis (ATCC 8387) was the organism used in this study of the biosynthesis of glycine. This organism is included in the large group of gas-producing lactic acid rods ordinarily characterized by a marked fermentation of pentoses, particularly arabinose.

Cultures of this organism were carried in 10 ml. of enriched agar medium (appendix A). Inocula for experimental use were prepared from these by transfer into 2 ml. of an enriched liquid medium (appendix A). Following incubation for a period of 12 to 18 hours at 37°C, cells were centrifuged and resuspended in 25 ml redistilled water. Often, it was found desirable to recentrifuge in order to reduce the extent of contamination by nutrients in the enriched broth. A drop of the final suspension was added to each assay tube with the aid of a sterilized syringe.

Assay Medium

The medium used in investigation of the biosynthesis of glycine was modification of that described by Dunn et al. (58), unless otherwise indicated, a substitution of all sodium salts with equimolar amounts of potassium salts was made, since our laboratory had obtained improved growth of the assay organism on this potassium medium (43). Furthermore, Mills, et al. (59) indicated that in studies with Lactobacillus arabinosus both unfavorable and favorable effects of sodium medium have been reported. Medium as used are constituents of those shown in Appendix B.

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[1] Identified as Streptococcus equirus by C. S. McClesky, J. Bact. 64, 140, (1952)

Experimental Methodology

The general procedures employed were essentially those of Henderson and Snell (15). Racks containing sixty tubes, with six tubes per row, in 10 rows was employed in all of these studies. The limiting amino acid or the limiting nutrient was added to every tube by means of an automatic dispersor such that each tube in a row received respectively: 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml. Water was then added so that the total volume in each tube was 1.0 ml. Then 1.0 ml of the double strength of medium was added to give a final volume of 2.0 ml. For precision and accuracy within the experiments, each tube was prepared at least in duplicate, and sometimes even greater replication was employed.

Sterilization

Toennics and Gallant (60) indicated that bacterial growth, as reflected in net acid production, is adversely affected by extended heating of the medium, thus suggesting that local variation in the intensity of heat sterilization may be causes of low reproducibility. Similar observations have been reported by Hill and Patton (61). In the experimental procedure, as a result of these and other findings, the steam pressure of the sterilizer was kept to 15 pounds for not more than 5 minutes (121°C). This procedure has proved to be very adequate. Following sterilization, the steam was cut off and the pressure was released to atmospheric pressure within one minute. The sterilized racks were allowed to cool at room temperature before inoculation was applied.

Incubation and Growth Measurement

The inoculated tubes were incubated in a constant temperature of

27 ± 1°C. Following 60-70 hours of incubation, all the racks were removed from the incubator for titration. A titrimetric method was used to determine quantitatively the acid produced in each tube by the assay organism during its growth, thus giving an index of growth. The acid produced was electrometrically titrated with approximately 0.05 N KOH. A quinhydrone electrode was used as the indicator electrode and a normal calomel electrode served as the reference electrode. The zero point of the galvanometer was set while the quinhydrone electrode was immersed in a pH 7.3 buffered solution. The amount of base required to complete titration of each tube was determined by means of an automatic titrator (62). Titrations were recorded as titration counts, and one hundred counts were the growth equivalent to approximately 4 ml of 0.05 N lactic acid (i.e. 0.2 milliequivalent of acid produced).

CHAPTER III

SPECIFIC STUDIES OF FACTORS AFFECTING GLYCINE BIOSYNTHESIS IN LACTOBACILLUS BREVIS

Review of Previous Investigation

The biosynthesis of serine by various microorganisms has been reviewed by Shive (63), Wood (64) and Lascelles, Cross and Wood (65). Evidence for the conversion of glycine to the alpha- and carboxyl carbons of serine in rat-liver homogenate has been obtained by Winnick, Moring-Glaesson and Greenberg (46) and evidence for the existence of the same reaction in livers of rats has been given by Sakami (51). Sakami (51, 67) also found that formate, which had been administered to the rats along with glycine, served as a source for the beta-carbon of serine; the possibility, that this incorporation was brought about via CO_2, was excluded. Sakami further suggested (67) on the basis of these and other studies that the conversion of glycine to serine was a reversible process.

$$HCOOH + CH_2NH_2COOH \rightleftharpoons CH_2OHCHNH_2COOH$$

The simultaneous incorporation of the two substances does not necessarily imply, however, that the incorporation is due to a condensation between glycine and formate or even between some derivatives of these. The one carbon fragments from the two substances might be incorporated by an entirely independent process. It was also reported

that the conversion, *in vivo*, of labile methyl groups and of the alpha-carbon of glycine to the beta-carbon of serine (66) was observed. This latter conversion was also observed *in vivo* by Siekevitz *et al.* (68). They also obtained evidence that the transformation of the alpha-carbon of glycine into the beta-carbon of serine proceeded via formate with rat liver slices.

Glycine replaced serine for the growth of mutant strains of *Escherichia coli* (69), indicating that glycine may be a precursor of serine, as it is in animal tissue. Growth experiments with lactobacilli also provided evidence, again indirect, that PGA[1] is required for the conversion of glycine to serine with *Leuconostoc mesenteroides P-60* (17) on media containing glycine but not serine. A function of vitamin B_6 in some stage of serine formation has also been indicated by replacement experiments with growing culture of *Streptococcus faecalis R*, *Lactobacillus arabinosus* and *Leuconostoc mesenteroides P-60* (36).

The growth experiments with lactobacilli give no clue as to whether or not serine is formed directly from glycine. With growing cultures of *Streptococcus faecalis R* exogenous glycine is not required for the synthesis of serine(70); in this case, the carbon skeleton must either come from some constituent of the growth medium other than glycine or result from some degradation products of glucose metabolism.

Again with reference to vitamin B_6, both cell suspensions and growing cultures of *Streptococcus faecalis* were found (65) to absolutely require pyridoxal for the formation of serine from glycine, but in recent work with partially purified extracts of pigeon liver, Kisluick *et al.* (71) and Blakely (72) have found no stimulation by pyridoxal phosphate; they suggest that this coenzyme may be strongly bound to the

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1 Pteryl Glutamic Acid

protein. Metzler et al. (73) have obtained a non-enzymatic synthesis of serine by heating together glycine, formaldehyde, pyridoxal and metal ions (Al^{+++}). They proposed an initial combination of glycine and pyridoxal with formation of a Schiff base which then reacts further with formaldehyde. If, as they also suggest, the enzymatic mechanism is similar - an attractive hypothesis - the high concentration of glycine required may be due to a relatively low affinity of glycine for the B_6 prosthetic group contained in the Schiff base, which may be an intermediate in glycine biosynthesis. Elwyn et al. (74, 75) also have demonstrated that the one carbon fragment formed from serine is at the oxidation level of formaldehyde rather than formate. The formulation of the biosynthesis of serine as a process involving a condensation between the alpha-carbon of glycine, activated by the Schiff base formation with pyridoxal phosphate (77) and tetra-hydro folic acid is reminiscent of the Mannich reaction (78). It was believed that the beta-carbon atom of serine is released as a coenzyme bound one carbon fragment unit at the oxidation level of " active formate"(57,56). Another hypothesis, that biosynthesis of serine involved formation and reduction of the enol form of ethyl formyl hippurate (79,46,66,80), is of considerable interest. This compound would be enolized readily so that serine biosynthesis may involve the enzymatic reduction of the enol form of amino malonic semialdehyde or a closely related compound of transient nature. These findings, together with the demonstration of an incorporation of formate-carbon into glycine, raises the question of whether formate is normally produced in intermediary metabolism. it should be pointed out that this formate would not function as one carbon source in lactobacilli.

The stoichimetry of this reaction in liver preparation is well known (82, 81, 72). This pathway has not only been useful in explaining biosynthesis of glycine in mammalian tissue, but also may occur in lactobacilli.

$$CH_2OHCHNH_2COOH + H_2O \longrightarrow CH_2NH_2COOH + CH_2(OH)_2 \quad (1)$$

$$CH_2(OH)_2 + 2O_2 \longrightarrow CO_2 + 2H_2O_2 \quad (2)$$

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad (3)$$

(+

$$CH_2OHCHNH_2COOH + O_2 \longrightarrow CH_2NH_2COOH + CO_2 + H_2O$$

Equation (1) and (3) are catalyzed by serine by hydroxymethylase and catalase respectively. It is seen that the two step oxidation to CO_2 takes place according to the net reaction, thus each micromole of serine disappearing should comsume two micromoles of oxygen and produce one mole of each of glycine and CO_2.

Introduction to Present Investigation

This investigation was undertaken to study further the biosynthesis reported by Schmidt in 1954 (83). Under certain experimental conditions serine synthesis in this organism occurs readily (Fig. 1) Lactobacillus brevis requires a high level of glycine for maximum growth in a serine-free medium. This high requirement for glycine arises from its involvement in the synthesis of serine. In other words, glycine serves as the major precursor for the biosynthesis of serine. It was further found that this synthesis was inhibited by alanine. Subsequently, it was shown that the alanine inhibition was relieved by glycine and it was suggested that the alanine interference in serine synthesis may be the result of an inhibition of glycine utilization.

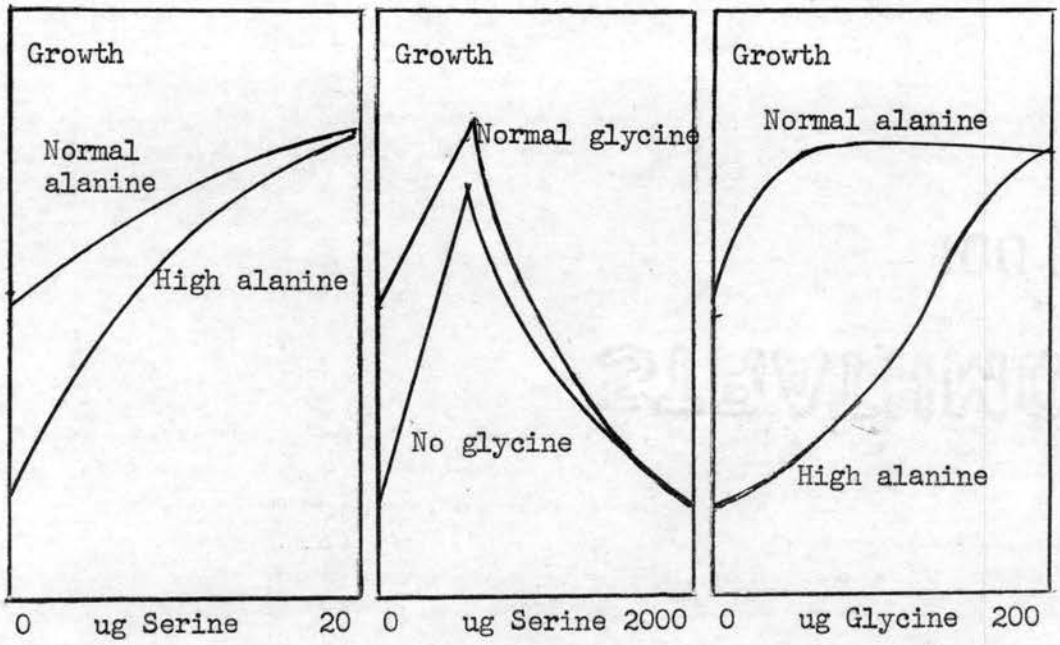


Figure 1 Figure 2 Figure 3

The curves shown in Figures 1-3 represent typical growth responses in serine-free media under varying conditions involving some of the effective factors influencing serine synthesis. The degree of synthesis is readily seen by inspection of the growth response in the blank (zero concentration) tubes. Thus, in Figure 1, a lower blank in the presence of high alanine indicates the inhibition of serine synthesis. The growth response to added serine is also seen. In Figure 2, serine synthesis is also indicated, but another effect is demonstrated, this being an inhibition of growth by relatively high amounts of serine. The effect of glycine in this situation is also shown, and this latter effect is further demonstrated in Figure 3.

Effect of Various Amino Acids

It is known that other amino acids may be involved either in stimulation of growth or in inhibition of growth. Hutchings and Peterson (84)

reported that alanine is able to counteract the inhibitory effect on growth of histidine and isoleucine, producing a combination which has a marked stimulatory effect. Camien and Dunn (85) have shown that glycine and serine also have an effect on the utilization of alanine by Lactobacilli. The effect of added alanine will also be influenced by the quantity of these amino acids in the medium. The inhibition of alanine on the synthesis of serine is worthy of attention. McCoy et al. (86) have shown that increasing concentrations of alanine will inhibit the growth response of Streptococcus faecalis R. Snell et al. (38) reported that serine inhibited the utilization of alanine and that this antagonism possibly was reversible. Previous reports from our laboratory (17, 83) have also indicated that alanine probably inhibited the synthesis of serine rather than the utilization of it.

The Lactobacilli vary widely in the kinds and amounts of amino acids required for optimum bacterial growth. Lactobacillus brevis, in our experiments, required 15 amino acids whereas Leuconostoc mesenteroides P-60 requires 17 amino acids. Dunn et al. (34) indicated that valine and glutamic acid are essential amino acids for 23 different strains of lactic acid bacteria.

Glycine Requirement of Lactobacillus Brevis

The glycine requirement of Lactobacillus brevis was described in an early report by Dunn et al. (34). In this paper it was shown that glycine was required for maximum growth of the organism, but that some growth occurred in the absence of glycine. In view of this report, it was decided to study this organism's ability to synthesize glycine. It was further felt that because of the ease by which the organism

synthesized serine from glycine that the reverse reaction, i. e., the synthesis of glycine from serine, might also occur and that this reaction might be altered by some of the factors which had been shown (83) to affect serine synthesis.

Results of some preliminary studies which provide some information as to the nature of the glycine requirement are shown in Figures 4 and 5. It is shown in Figure 4 that in the absence of glycine (zero concentration) an appreciable amount of growth occurs, indicating that glycine synthesis is taking place. The relatively high blank in the presence of high levels of alanine requires further explanation. It was at first felt that this growth may have been due to glycine contamination in the medium. However, this is rather unlikely since the constituents of the medium support essentially no growth of Leuconostoc mesenteroides in a glycine-free medium. Therefore, this must be explained on some other basis, and a limited capacity to synthesize glycine, even in the presence of high alanine, appears to be such a basis. Reference to Figure 5 indicates a similar level of growth in the absence of both glycine and serine. Thus, a limited capacity to synthesize both glycine and serine in the absence of both of these amino acids must be postulated, these syntheses both involving precursors in the medium other than serine and glycine.

Further study of the data presented in Figure 4 clearly indicates a depressing effect of alanine in glycine utilization, this effect occurring in the presence of serine. This inhibitory effect of alanine is also seen in Figure 5, in which alanine appears to also inhibit serine utilization.

Figure 4. Inhibitory Effect of Alanine on Response to Glycine

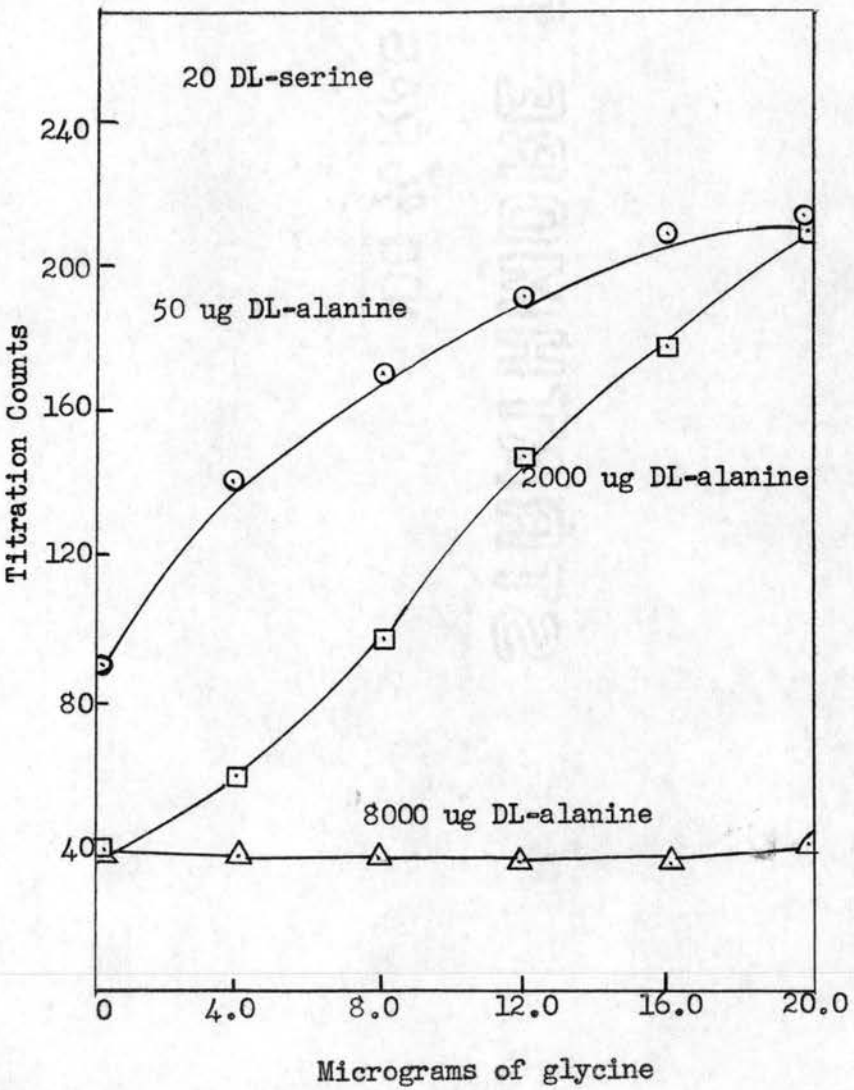
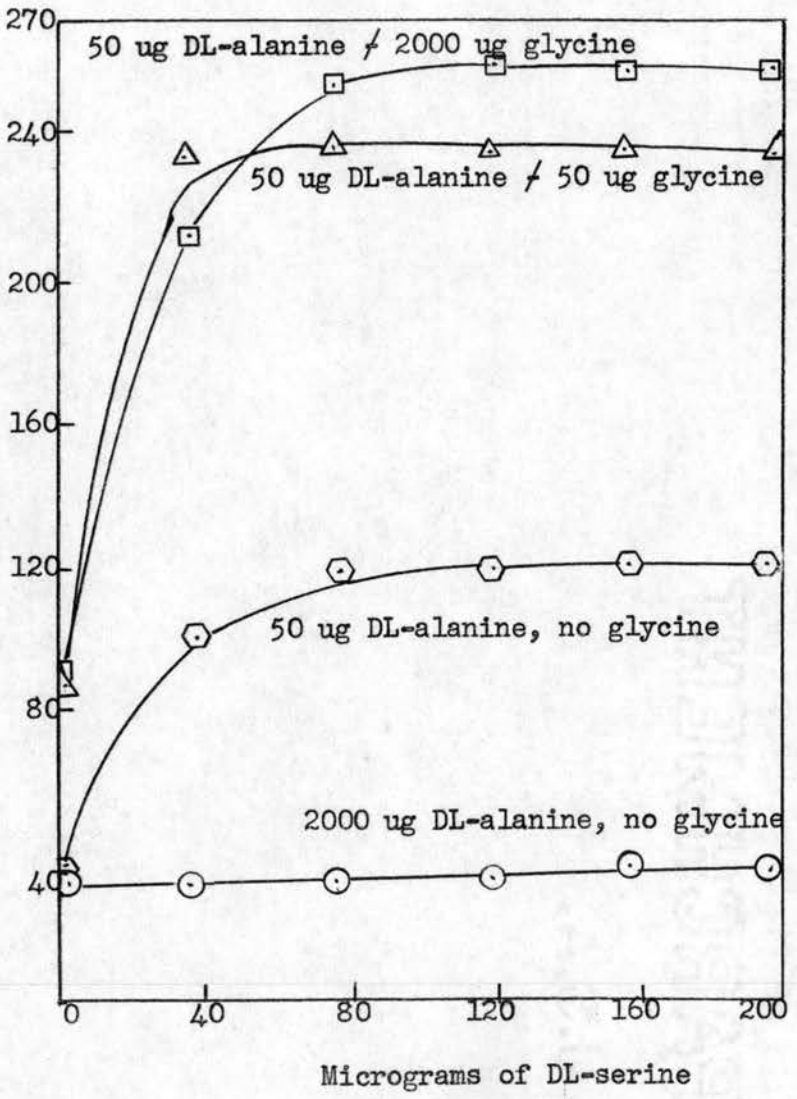


Figure 5. Inhibitory Effect of Alanine on Response to Serine in Presence and Absence of Glycine



Another aspect of the glycine requirement of this organism is shown in Figure 5. It is seen again that a limited amount of growth occurs in the absence of glycine; however, this growth is not increased by increasing amounts of serine, suggesting that regardless of the amount of alanine in the medium the organism can not synthesize an adequate amount of glycine for maximum growth.

Serine as a Possible Precursor of Glycine

The importance of glycine in animal and plant metabolism as a precursor of many nitrogen compounds, such as creatine, porphyrins chlorophyll, uric acid and nucleic acid purines is now well-established (87). Relatively little is known, however, about the metabolic origin of this amino acid from nitrogenous precursors. That glycine can be synthesized by mammals has been recognized for almost half of a century (88) but conclusive proof that it is a nutritionally dispensible amino acid has been obtained only recently in feeding experiments with synthetic diets (89).

Metabolism of glycine is closely linked to that of serine, since both amino acids are readily converted into one another both in vivo (50, 51) and in vitro (72, 71), or two carbon compounds in sufficient amounts, and both mechanisms have, in fact, been proposed for the biosynthesis. More recently it has been shown that glycolic acid (90, 91, 92) can be used for the biosynthesis of glycine, presumably by reductive amination of glyoxylic acid.

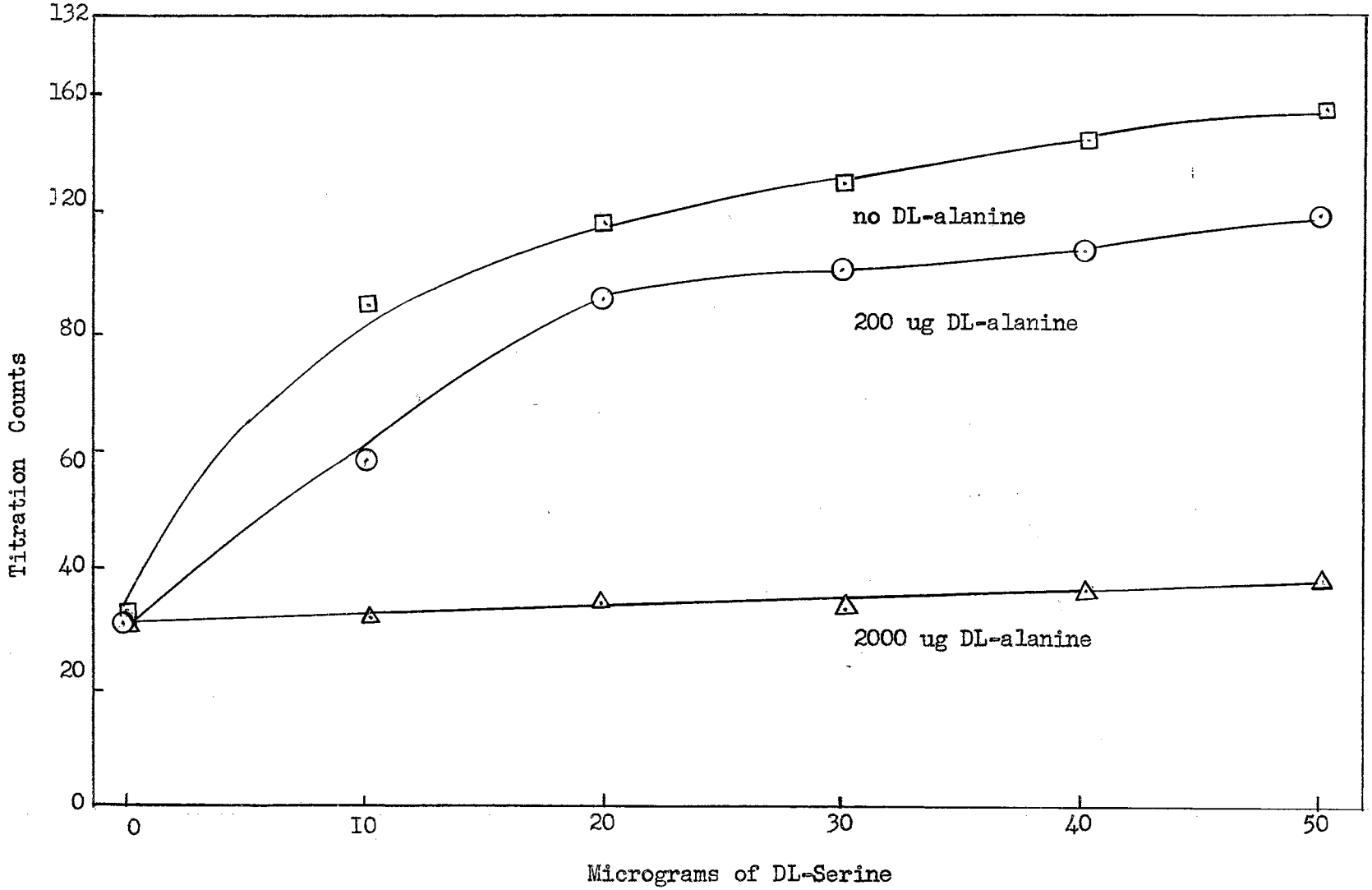
It is not clear, however, whether these C_2 acids are formed in sufficient amounts to account quantitatively for glycine biosynthesis. The available evidence concerning the relative importance of C_3 and C_2

precursors have so far been inconclusive. Many attempts have been made to determine the nature of the C_2 intermediate since it would be the key to the confirmation of glycine synthesis.

The maximum serine requirement for growth of Lactobacillus brevis both in the absence as well as the presence of glycine is approximately 40-50 ug DL-serine, as is seen in Figures 5 and 6. Increasing the concentration of serine above this amount is seen to promote no further growth, as is clearly shown in Figure 5. However, it is also seen in Figure 5 that maximum growth is not obtained unless glycine is added. It may be further pointed out that during the extensive experimentation conducted in the course of these studies, maximum growth in the absence of glycine was not obtained under any conditions tested. Thus, it would appear that the limited growth response to addition of serine is merely due to satisfying the organism's needs for serine per se for incorporation into protein.

Accordingly, the evidence for serine functioning as a precursor of glycine is not strongly supported by the results of these studies. The observations obtained suggest that the limited glycine synthesis which occurs may actually be from some other precursor, not yet identified, which is present in the medium or which is formed by certain reactions which may occur in the medium. Further evidence which suggests that serine is not a major precursor of glycine will be seen in subsequent figures, although it is felt that serine may play a contributory role in this synthesis. It should also be pointed out that these results do not conclusively eliminate serine as a possible precursor of glycine in in this organism.

Figure 6. Inhibitory Effect of Alanine on Response to Low Levels of Serine.



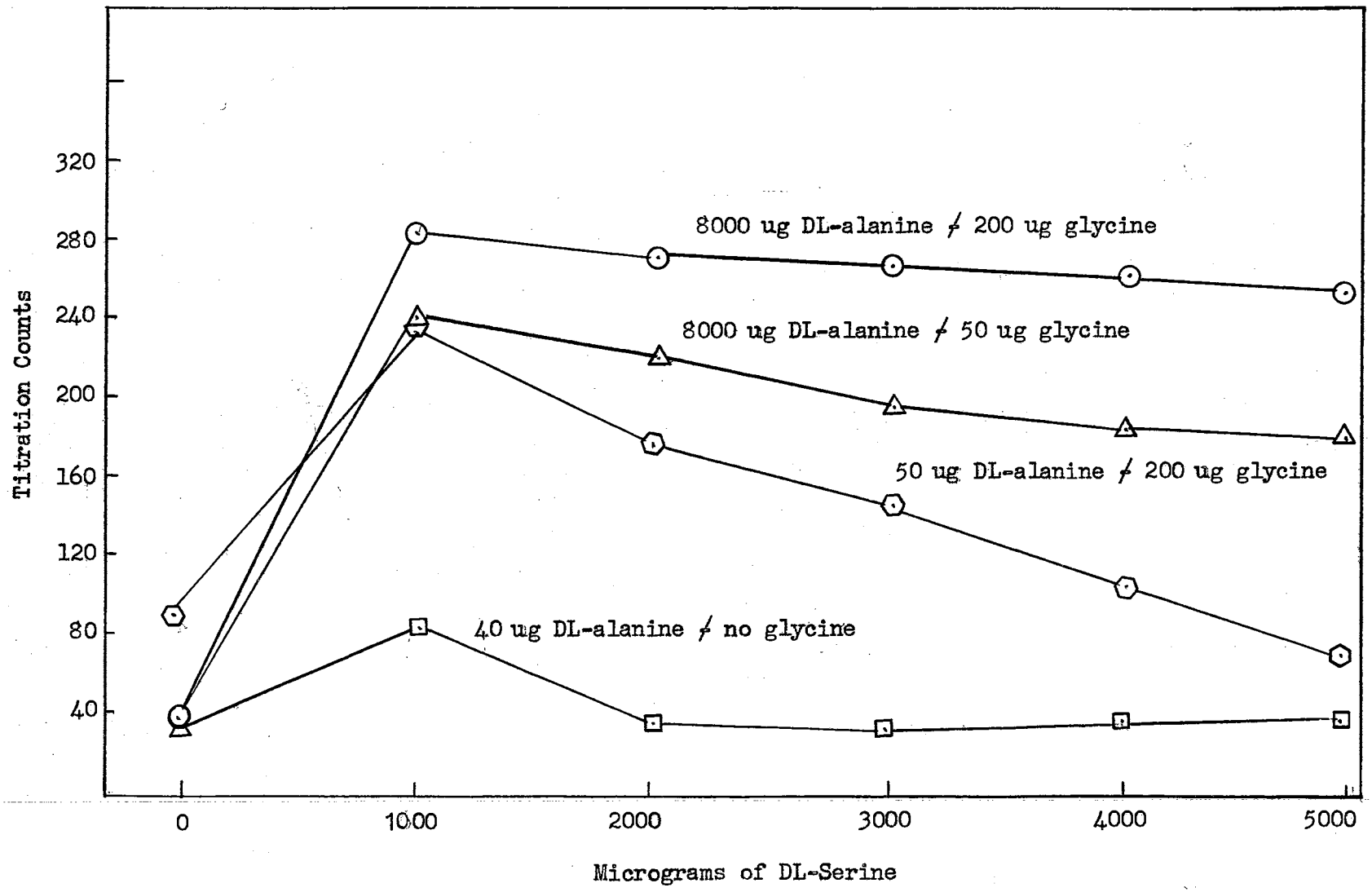
Serine Inhibition in Glycine Synthesis

Glycine toxicity in the chick has been observed (93) and it can be prevented by the administration of high levels of folic acid, but it is not known why glycine is toxic nor how folic acid enables the chick to metabolize excess glycine successfully. Furthermore, the specificity of glycine toxicity was demonstrated by the failure of serine to depress growth or to cause any symptoms of toxicity while growth depressing effects of alanine and tyrosine were not corrected by folacin. A number of investigators have observed a retardation in the growth of experimental animals fed certain diets in which the amount of a single amino acid has been shown to cause growth reduction of varying severity [methionine, glycine, valine, proline, arginine, leucine, lysine, phenyalanine, aspartic acid, cystine, isoleucine, threonine, tryptophan, glutamic acid. (94-100)]

An antagonistic effect of threonine on the utilization of serine in Streptococcus faecalis R (94) is also of interest; however, almost no published data has been found which deals with a deleterious influence and inhibition by serine on bacterial growth.

Despite the numerous studies that have appeared in the past decade dealing with the metabolism of serine and glycine in microorganisms, many aspects of the subject require further study. Particularly in this regard is the need for more information concerning the inhibitory aspects of serine in metabolism. In the present investigation, serine has been found to be very effective in producing an inhibition that can not be readily overcome with at least some of the vitamins or other amino acids studied, except under certain specific conditions which will be described later.

Figure 7. Inhibitory Effect of High Levels of Serine with High and Low Levels of Glycine and Alanine.



In a further attempt to implicate serine in glycine synthesis, experiments using relatively higher amounts of serine were conducted. The results of some of these studies are shown in Figure 7. As shown previously, it is seen again that the organism responds favorably to increasing amounts of serine and it is also seen that for maximum growth an appreciable amount of glycine must be presented. However, it was surprising to find that if the concentration of serine was raised above a certain level an inhibition of growth resulted. This inhibition is most clearly seen in the curve representing the results in which a relatively low amount of alanine and adequate glycine were present in the medium. In this curve, near-maximum growth is seen at 1000 ug DL-serine per tube, but above this concentration the growth obtained is progressively less until it becomes negligible at 5000 ug serine per tube.

Other interesting observations regarding this previously unrecognized inhibition may be made from the other curves shown in this figure. Alanine is seen to have a marked influence on this inhibition, appearing to have a stimulatory effect. At either of the two different levels of glycine, it is seen that 8000 ug alanine almost completely relieved the inhibition. It should be pointed out that results of all pertinent studies are not presented; however, further evidence of this effect of alanine will be presented later in Figure 10.

Effect of Threonine on Utilization of Serine

Threonine antagonism of the utilization of serine was reported by Meinke (94), and has become a rather well-known example of competitive inhibition among amino acids. Threonine has long been known to be an essential amino acid for mammals, and it is also an essential nutrient

for Lactobacillus brevis. Because of its close relatioship to serine, it was desirable to determine the effect of threonine on the utilization of serine in this organism.

A high amount of threonine added to the basal medium is shown in Figures 8 and 9 to cause a decrease in growth at subinhibitory levels of serine. It is seen in this figure, however, that at higher concentrations (inhibitory levels) of serine, threonine appears to function as a growth stimulant. These two effects may appear to be contradictory, but this may not seem to be too inconsistent if the stimulatory role of threonine is viewed as a stimulation which results from one inhibition relieving another inhibition. This explanation, though not proven, is very similar to thatgiven for the stimulatory effect of high concentrations of alanine, which was presented in the previous section.

The Alanine Inhibition of Serine Utilization

It is generally accepted that structual analogues of a given metabolite exert their stimulatory or inhibitory effect upon growth by virtue of their ability either to substitute for the metabolite in metabolism or to interfere with its normal utilization. By omitting a certain amino acid from the medium, growth can be made dependent upon its synthesis. The concept of competitive antagonism between metabolite and closely related antimetabolites first enuciated by Woods and Fildes (64) has been firmly established through experimental work.

It has been shown previously that the utilization of glutamic acid (21) by Lactobacillus arabinosus is strongly inhibited by relatively

Figure 8. Inhibitory Effect of Threonine on Response to Low Levels of Serine

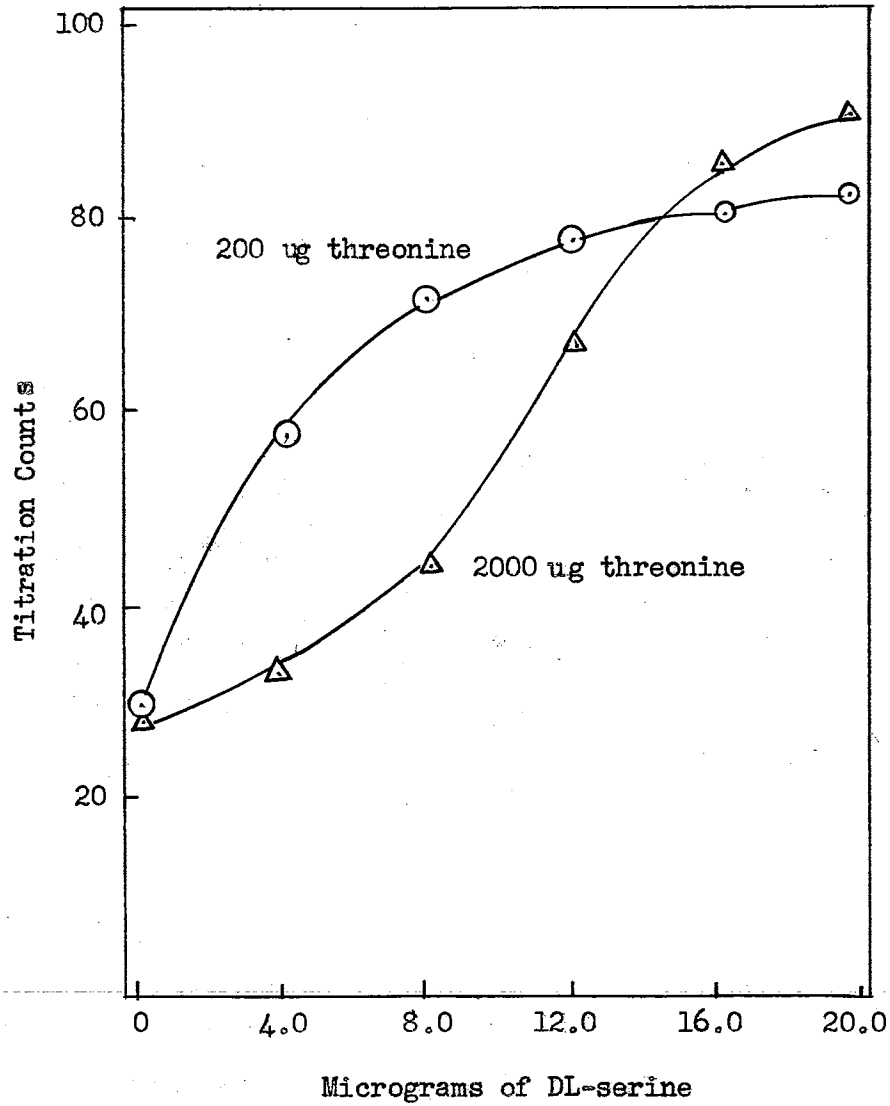
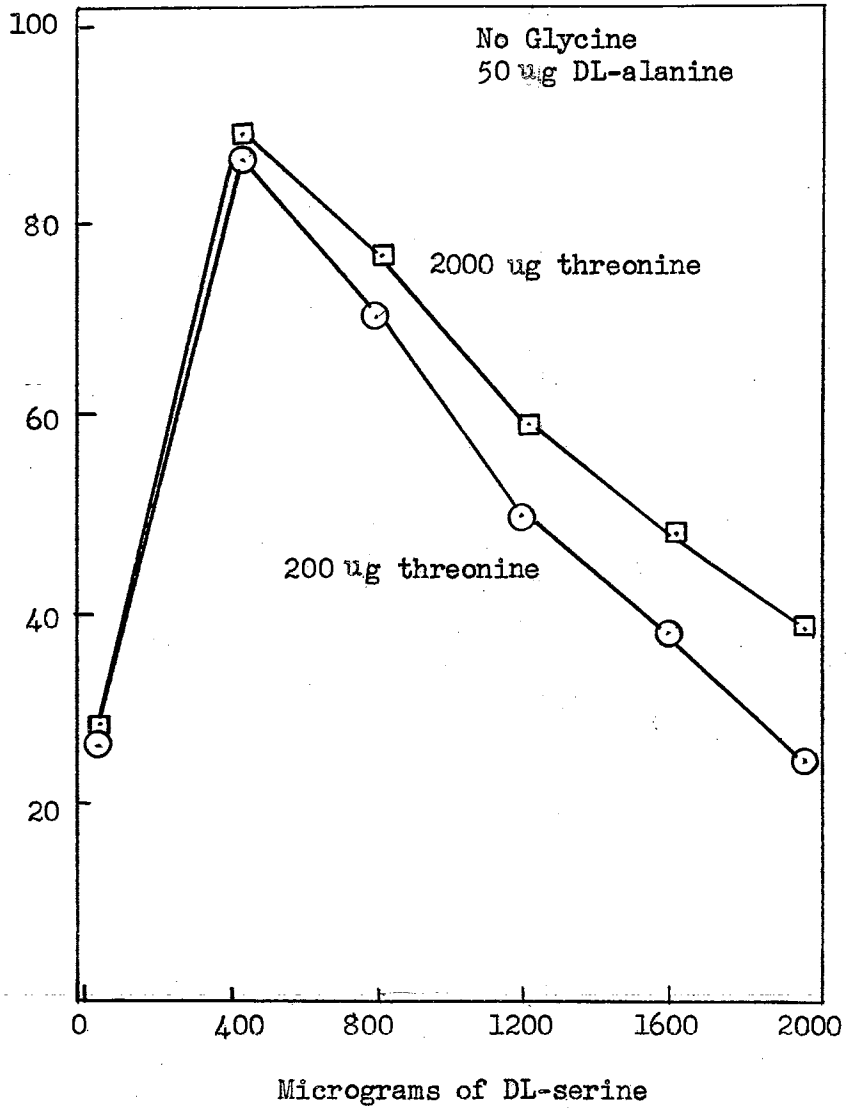


Figure 9. Stimulatory Effect of Threonine on Response to Inhibitory Levels of Serine

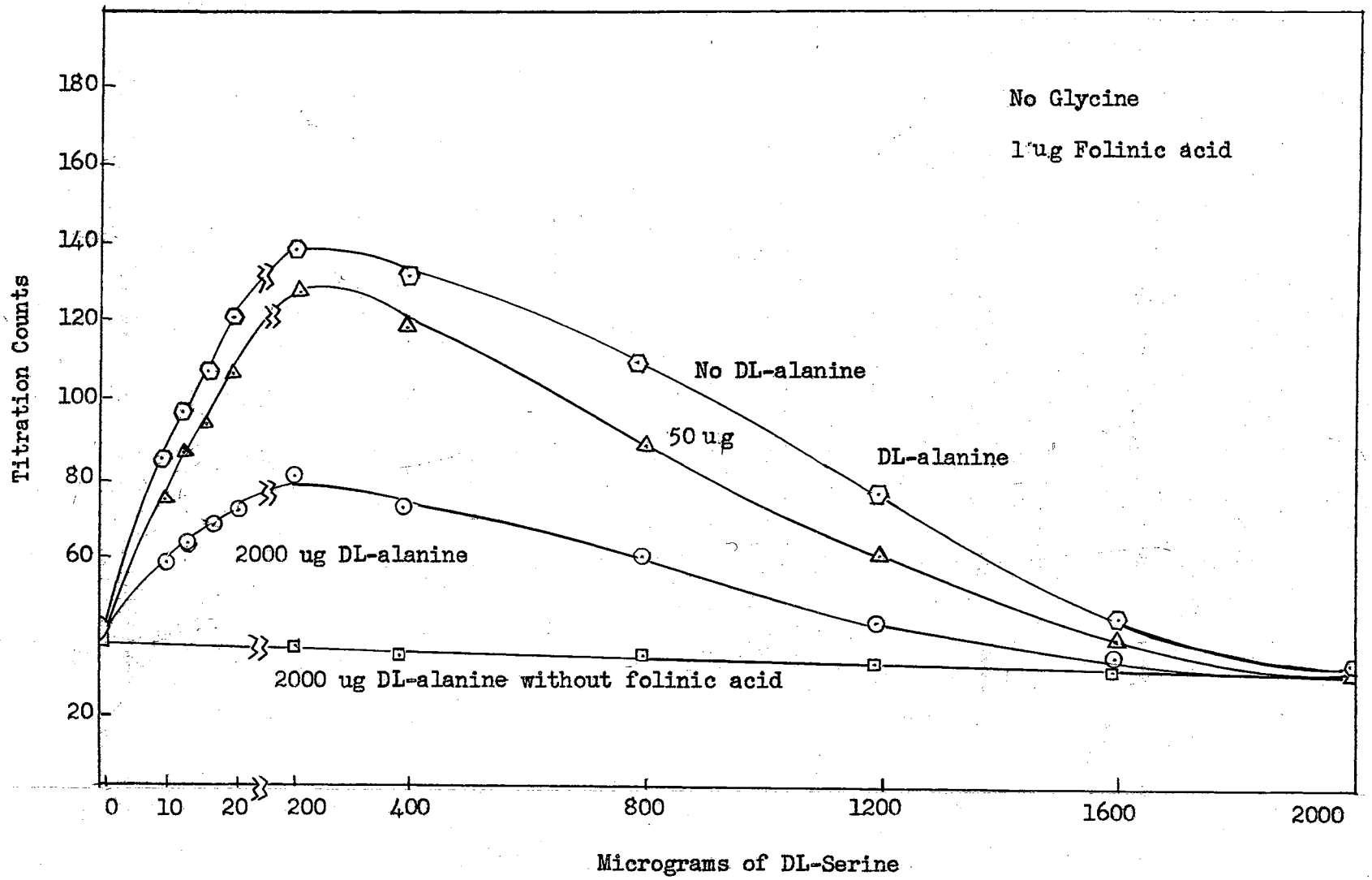


high concentrations of aspartic acid and threonine. Other workers (47, 89) have found that leucine and valine in moderate concentrations may interfere with the utilization of D-isoleucine by Lactobacillus arabinosus. Snell, et al. (38) have previously reported the inhibitory effect of glycine, serine, beta-alanine, and threonine on the utilization of alanine. Gladstone (101), using Bacillus anthracis, found valine is inhibitory to leucine utilization and vice versa. Similar interrelationships were also found for valine and threonine, and threonine and serine. For study of these interrelationships, Shive et al. (100) elaborated and extended the method of inhibition analysis.

Some of the observations which were made in the studies with respect to the alanine inhibition have been previously indicated in Figure 7. Alanine was seen to relieve the inhibition caused by high levels of serine under conditions in which glycine was also present in the medium.

Further studies conducted on the effects of alanine are shown in Figure 10. Under conditions requiring the synthesis of glycine (no glycine added to the medium), increasing amounts of alanine are seen to progressively reduce growth. When the conditions require, in addition, that the organism synthesize alanine as well as glycine the best growth is obtained. In response to increasing amounts of serine, growth improvement is again seen at the low levels and growth depression is seen at the higher levels of serine. However, there are points of difference between Figure 7 and this figure, both in the conditions and in the results obtained. It will be observed that under the conditions requiring synthesis of glycine, no growth is obtained, unless folinic acid is added. Folinic acid was not used in the previous experiment from which Figure 7 was obtained.

Figure 10. Inhibitory Effect of Alanine on Response to both Stimulatory and Inhibitory Levels of Serine.



An adequate explanation of these effects of alanine cannot be made on the basis of present studies. A possible explanation which is in agreement with these and other results obtained might be based on an inhibitory effect of alanine on glycine synthesis and of serine on glycine utilization. It is recognized, however, that because of the complex interactions between these three amino acids both from the standpoint of their synthesis as well as their utilization, that another explanation may be found to be true.

In an attempt to further elucidate the basic mechanisms involved in these interrelated inhibitions, it should be mentioned that some preliminary studies using inhibition analysis as described by Meinke (94) were conducted. The effects of alanine versus serine were tested over a limited range of concentrations and a fairly constant inhibition index was obtained. The results suggested that alanine was competitively inhibiting utilization of serine, but it is felt that further experimentation would be required for confirmation.

The Effect of Sodium and Potassium in Biosynthesis of Glycine in Lactobacillus brevis

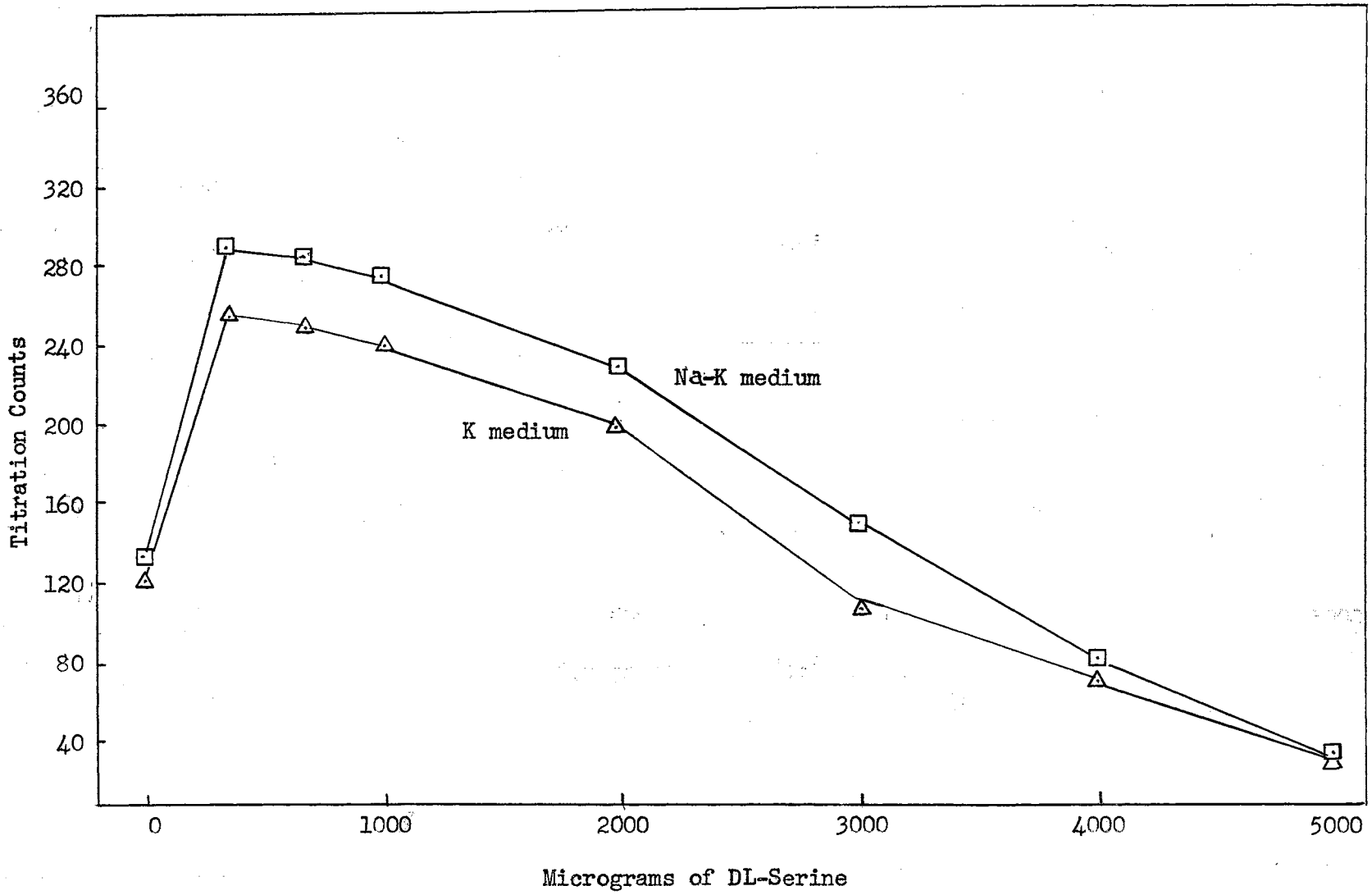
The nutritional requirements of any lactic acid bacteria can not be defined accurately without an evaluation of the interrelationships between the many chemical substances and physiological factors which are involved. It has been previously mentioned that there was great variation as to the amounts of the different amino acids necessary for growth. This variation is believed to be not only dependent on the growth factors in the medium, but also on the levels of the minerals as well as the amino acids.

On a medium deficient in potassium and relatively free of sodium and ammonium ions, it was found that proper potassium ion supplementation permitted excellent growth in five strains of lactic acid bacteria (102, 43) in which the sodium ion was toxic under ordinary conditions. Mills, et al. (103, 59, 49) have recently shown that varying the concentration of specific inorganic cations may produce a favorable effect of sodium on certain amino acid interrelationships as well as on bacterial growth. Furthermore, it was pointed out that the nature of potassium and sodium ion effects on amino acid interrelationships depends on the ammonium ion concentration as well as the pH of the medium.

The requirements for sodium and potassium in lactic acid bacteria are most complex, and this problem has had less attention than amino acid interrelationships. The main reason for these limited studies is undoubtedly connected with the difficulty involved in producing media sufficiently free from traces of mono- and divalent ion contaminant to allow study of the ion requirement. The present study was designed to see the effects of sodium and potassium ions on biosynthesis of glycine in Lactobacillus brevis, particularly since under certain conditions a favorable effect of the sodium ion has been observed with Lactobacillus arabinosus in interrelationships involving the three major amino acids studied in this investigation.

The results appear to support the observation that the sodium ion under certain conditions favorably affects the synthesis of serine, as shown in Figure 11. It seems also highly probable that the sodium ion is a nutritionally favorable ion for utilization of serine for bacterial growth. The magnitude of the serine inhibition, as indicated in Figure 11, is about the same as has been previously described; this figure,

Figure 11. Stimulatory Effect of the Sodium Ion on Response to both Stimulatory and Inhibitory Levels of Serine



then, also provides further evidence of the serine inhibition in this organism.

Other Related Studies

Glycine biosynthesis depends on how one can minimize the inhibiting effects of the other amino acids previously mentioned. Studies of other factors affecting this synthesis are therefore complicated by the interrelationships existing between the amino acids and the other factors under consideration. Some studies have been conducted in folinic acid, and an improvement of growth over that supported in the presence of folic acid was obtained. However, because of the complex nature of this investigation, the effect of folinic acid could not be clearly explained in terms relating it to any one specific function. It is desired, nevertheless, to point out that this effect was observed, and that it is in contrast to previous observations made with this organism in which folinic acid was found not to affect the biosynthesis of serine. Further investigation of this effect is warranted.

CHAPTER IV

GENERAL DISCUSSION

A large number of publications have appeared on the microbiological assay technique for amino acids and vitamins, and some of the fundamental concepts of biochemistry have arisen from studies of microbiological nutrition, of bacterial enzyme action, of amino acid interrelationships and pharmacological antagonisms of cells. Along the path of its development, it has been the contributions from many relatively narrow, specific investigations that have determined the direction of research and provided the framework upon which subsequent experimental observations have been fitted together permanently or until a better mechanism could be elucidated to explain them. The present investigation was designed to see more distinctly how certain amino acids were interwoven in the biosynthesis of glycine. As measured in terms of bacterial growth the biosynthesis of glycine was retarded by the action of alanine, and the synthesis as well as utilization of serine was also inhibited by the alanine. Thus dual alanine inhibition of both serine and glycine synthesis in Lactobacillus brevis is of particular interest from the standpoint of the metabolism of one carbon intermediates in biochemistry. Of further pertinent interest was the finding during the course of these studies that serine also possessed inhibitory properties at essentially the same concentrations at which the alanine inhibition occurred. It was apparent from these studies that serine, like

alanine, was able to function both as a growth-promoting amino acid and as an amino acid antagonist.

The mechanism by which serine inhibits the growth of Lactobacillus brevis is not entirely clear, but certain observations, in addition to the original recognition of this inhibition, have been made. It should be pointed out, first of all, that this inhibition by serine occurs in an otherwise complete medium. However, the nature of the inhibition is substantially altered when glycine is omitted from the medium, i. e., when the organism is made to synthesize its own glycine. In the glycine-free medium, alanine functions as an agent which increases the magnitude of the inhibition, but in the complete medium alanine relieves or overcomes the inhibition by the serine. A possible explanation for these contrasting observations might be made on the basis of alanine functioning as an antagonist of serine in both cases. In the former case (glycine-free medium) alanine might conceivably be interfering with some phase of the glycine-synthesizing system and would, thus, contribute to an inhibition. However, in the latter case (complete medium) alanine could be visualized as antagonizing the inhibitory effect of serine on some other metabolic system, possibly that of glycine utilization, and the net effect of this latter antagonism of alanine on the serine inhibition would be a growth stimulation. This explanation would be particularly attractive if serine also served as a precursor of glycine and if, in high levels, it interfered with glycine utilization. The former possibility has neither been eliminated nor proven on the basis of these studies, but considerable evidence has been obtained for the latter possibility.

With respect to the precursor(s) of glycine, it has not been

possible to positively implicate serine in this role. This may have been due, in part, to the limited capacity of the organism to synthesize glycine, since in no experiment was maximum growth obtained on a glycine-free medium. This limited capacity, in turn, may have been due to at least two possible causes, one being that some precursor other than serine was present in the medium in an inadequate amount and the other being that some cofactor, not identified, for glycine synthesis from serine was present in a limiting amount. Either of these two latter possibilities, together with the complicating early appearance of serine inhibition made a convincing demonstration of a precursor function for serine difficult to prove by use of the techniques employed in these studies.

From all these studies, the degree of synthesis and utilization of certain amino acids in Lactobacillus brevis is clearly a function of other amino acids and of other constituents in the medium. Therefore, it must be kept in mind, for further study, that careful control of these factors is very important. It is felt that an improved procedure such as statistical method of inhibition analysis might be employed to study the antagonisms between these amino acids in order to elucidate the fundamental mechanisms which are involved.

CHAPTER V

SUMMARY

The present study was undertaken to determine the nature of glycine biosynthesis in Lactobacillus brevis, and to investigate some of the factors, particularly related amino acids, which might affect the synthesis. Bacterial growth as measured by titration of lactic acid produced was used as the criterion for evaluation of the effects of the different factors. When it was discovered that serine markedly inhibited growth of this microorganism, more extensive studies were conducted on factors affecting this inhibition by serine. Alanine, especially, was also studied in regard to both glycine synthesis and serine inhibition.

Lactobacillus brevis was found to have a limited capacity for the synthesis of glycine under the various conditions employed. Alanine was found to depress this synthesis, and serine also was found to have an effect. Attempts to implicate serine as a precursor of glycine in this microorganism were not convincing, though a stimulatory effect of low levels of serine in a glycine-free medium was observed.

The finding of most interest in these studies was that while serine was stimulatory at low levels, it was very inhibitory at higher levels. This inhibition by serine was found not to be a general toxicity, but rather a specific inhibition which was related to the concentrations of other amino acids in the medium. Glycine concentrations altered the degree of inhibition, and it was suggested that the inhibition by serine

was due to an interference with glycine metabolism. Alanine also had a striking effect on this inhibition, and was found to be either stimulatory or inhibitory, dependent on whether or not glycine was also present in the medium. Other related factors were also studied and possible explanations of the effects of the various factors studied have been discussed.

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APPENDIX

A. Enriched Media for Storage and Transfer of Lactobacilli.

Agar medium:

Agar	15.0 gm
Yeast extract	10.0 gm
$KC_2H_3O_2$	5.0 gm
Glucose	2.5 gm
Water to 1000 ml	

Liquid transfer medium: (for 100 tubes at 2.0 ml per tubes)

Arabinose	0.7 gm
Glucose	0.3 gm
K-citrate	0.1 gm
K_2HPO_4	0.5 gm
Tryptone	0.5 gm
NH_4Cl	0.3 gm
K-acetate	1.0 gm
Yeast extract	0.5 gm
Vitamin solution[1]	0.5 ml
Salt S solution[2]	1.0 ml
Dissolved in water, and adjusted pH at 6.0.	

The media were sterilized and stored in the refrigerator.

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[1] Composition given in appendix B

[2] Composition given in appendix B

B. Basal Media for *Lactobacillus brevis*

Amino acid mix (for 100 tubes at 2.0 ml assay volume):

DL-Aspartic acid	200 mg	DL-Tryptophan	40 mg
DL-Alanine	200 mg	DL-Valine	40 mg
L-Glutamic acid	200 mg	L-Histidine HCL	40 mg
L-Arginine HCl	40 mg	L-Cystine	20 mg
DL-Isoleucine	40 mg	Glycine	20 mg
DL-Methionine	40 mg	L-Leucine	20 mg
DL-Phenylalanine	40 mg	L-Proline	20 mg
L-Lysine HCl	40 mg	L-Tyrosine	20 mg
DL-Serine	40 mg	Made up to 25 ml with HCl	
DL-Threonine	40 mg	and heat.	

Sugar mix for *Lactobacillus brevis* (for 100 tubes at 2.0 ml assay volume)

L-Arabinose	4.0 gm
$KC_2H_3O_2$	4.0 gm
K_2HPO_4	1.0 gm
NH_4Cl	0.6 gm
Glucose	0.6 gm
Modified salts S soln.	2.0 ml
Vitamin soln.	2.0 ml
AGU-soln.	2.0 ml
Xanthine soln.	2.0 ml

Twenty-five ml of amino acid mix added, the total volume made up to 100 ml and the pH adjusted to desired value.

Solutions for the preceding sugar mix:

Modified Salts S

$MgSO_4$ $7H_2O$	4.0 gm
$MnSO_4$ $7H_2O$	0.2 gm
$FeSO_4$ $7H_2O$	0.2 gm

Dissolved in water and made up to 100 ml.

AGU-solution

Adenine SO_4	250 mg
Guanine HCl	250 mg
Uracil	250 mg

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

Xanthine solution

Xanthine	250 mg

Dissolved in dilute KOH and made up to 250 ml.

Vitamin Solution

Ca-pantothenate	25.0 mg
Niacin	25.0 mg
Riboflavin	25.0 mg
Thiamin	25.0 mg
PABA	5.0 mg
Pyridoxal	5.0 mg
Biotin	0.25 mg
Folic acid[1]	0.25 mg

Riboflavin dissolved first in hot H_2O and HCl, other vitamins added, and total volume made up to 250 ml.

---

[1]Folic acid stored separately in dilute KOH in 50% ethanol, 2.0 microgram/ml.

VITA

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Master of Science

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