THE UTILIZATION OF FREE AND PEPTIDE-BOUND AMINO ACIDS BY LACTIC ACID BACTERIA

By

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PREFACE

The purpose of this investigation was to study the different growth-responses of bacteria when amino acids are supplied in the external medium in free form or in peptide-bound form. Why do free amino acids and peptides of the same amino acids cause a difference in growth-response? This investigation was initiated in order to answer this question on the basis that entry of the amino acid or peptide into the cell may be a significant factor in growth-responses. Although this study does not provide data concerning uptake of the peptide-bound amino acids into the cell, a sufficient amount of groundwork has been laid in order to continue a well-systemized study of the utilization of free and peptide-bound amino acids.

I am gratefully thankful for the facilities and financial assistance in the form of a Research Assistantship which was provided me by the Department of Biochemistry of the Oklahoma State University. I am also thankful to the faculty of the Department of Biochemistry for their help and cooperation in supplementing my graduate study with their attitudes and philosophies of research and teaching in the field of chemistry.

I wish to express my gratitude to Dr. E. A. Grula of the Department of Bacteriology for his occasional assistance in use of equipment and his helpful suggestions which enable my better use of valuable time.

Most of all I am sincerely grateful for the helpful guidance and suggestions of my adviser, Dr. R. J. Sirny, Associate Professor of

Biochemistry. His advice and philosophy have meant more to me in the field of science and understanding than just merely in the writing of this thesis and directing a research problem. My highest acknowledgement to Doctor Sirny is that scientists who are aware and concerned of people as he definitely is truly give chemistry a human nature which I believe is vitally needed among the science teachers of higher education.

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CHAPTER I

INTRODUCTION

The utilization of free and peptide-bound amino acids by lactic acid bacteria has been studied in relation to inhibitors which are present in the growth media. The dose-responses involving the utilization of glutamic acid by Lactobacillus plantarum have shown that aspartic acid inhibits the utilization when lower concentrations of glutamic acid are present. The growth-response to lower doses of glutamic acid can be raised by substituting asparagine for aspartic acid in the growth media.

The mechanism involved in the low dose-response in the presence of aspartic acid and the higher dose-response with asparagine has not been definitely established by experimental evidence. One purpose of this investigation was to show the amount of glutamic acid which is incorporated into the interior of a resting cell of Lactobacillus plantarum under "normal" and inhibitory conditions. From these studies a clue might be found as to whether or not the cell wall or membrane plays a role in the inhibition of glutamic acid utilization by aspartic acid. Additional studies showed a relationship between the lag phase in the response of Lactobacillus plantarum to glutamic acid and the uptake of glutamic acid by resting cells of Lactobacillus plantarum in the presence of aspartic acid.

A survey was made of the utilization of the peptide-bound amino acid (glycyl-L-glutamic acid) in the presence of aspartic acid and aspar-

agine. When the amino acid is available to the microorganism in a peptide-bound form, a lower dose-response is not always observed under inhibitory conditions as is observed when the amino acid is available in its free form. The differences in the dose-responses between the peptide-bound form and the free form will be discussed.

CHAPTER II

THE UTILIZATION OF GLYCYL-L-GLUTAMIC ACID BY SEVEN LACTIC ACID BACTERIA

History

Many bacteria require certain amino acids for their proper nutrition. When the amino acid is supplied in peptide-bound form, the growth-response is sometimes increased over that of the free amino acid. Woolley first described this increased growth as "strepogenin" activity. This term has commonly been interpreted to mean activity due to the presence of peptides; however, Sprince and Woolley (1) used the term to designate an unidentified factor which is necessary for the "early" growth of certain bacteria. Although this factor is present in partial hydrolysates of proteins, "strepogenin" as used in this investigation describes a stimulatory growth-response and not a particular peptide. Peptides have recently been found which have strepogenin activity; thus, from insulin a peptide, L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid, was isolated and synthesized and found to possess this activity (2). A peptide containing fourteen amino acids was found to be stimulatory for Lactobacillus casei (3) as well as a peptide isolated from hydrolysates of oxytocin (4). Peters and Snell (5) have showed that a strain of Lactobacillus delbrueckii has strepogenin activity with peptides containing fourteen amino acids, but smaller peptides, glycyl-L-tryptophan and glutathione, are inhibitory to growth. Even though some partial hydrol-

ysates contain strepogenin activity, Merrifield (6) has reported a competitive inhibition of the utilization of peptide-bound amino acids by the peptides themselves in Lactobacillus casei.

As strepogenin activity implies, the peptide is utilized at a higher rate and total amount than the respective free amino acid. Demain and Hendlin (7) have recently showed that a mutant of Bacillus subtilis has a higher rate of growth with partial enzymic hydrolysates of casein than with the complete acid hydrolysates. They also found that di-, tri-, and tetra-glycine peptides decrease the lag phase and increase the growth rate. Some instances are noted in which the peptide is able to overcome the antagonisms of amino acid pairs in Leuconostoc mesenteroides (8). In every case the peptide containing one of the antagonistic pairs is more effective in relieving the antagonism than the free amino acid. Kihara and Snell (9) have shown that peptides are more effective in overcoming the inhibition induced by ethionine in the utilization of methionine in Leuconostoc mesenteroides. Other peptide effects were observed in other organisms with beta-2-thienylalanine or canavanine as inhibitors.

Since Lewis and Olcott (10) first reported the inhibition of the utilization of glutamic acid by aspartic acid in Lactobacillus plantarum, investigations have been made in order to study the inhibition and overcome the inhibitory effect by substituting asparagine for aspartic acid. Based on the earlier work of Henderson and Snell (22), optimal conditions for glutamic acid utilization in the presence of aspartic acid were further studied by Clabaugh (11) for Lactobacillus plantarum. Ravel, et al. (12) overcame the aspartic acid inhibition by addition of glutamine or higher concentrations of glutamic acid. Sondheimer and Wilson (13) found that the utilization of acidic glutamic acid peptides is increased at

lower pH values, but neutral glutamic acid peptides are not affected. They used pH 6.5 as the optimum for the study of glutamic acid accumulation in cells of Streptococcus faecalis and Staphylococcus aureus to explain the growth-response to the peptides in Lactobacillus plantarum; however, the uptake of glutamic acid into the cells was not determined in Lactobacillus plantarum.

Various mechanisms for explaining the increased activity of the peptide over its respective free amino acids have been proposed. Among these are prior hydrolysis of the peptide before entering the cell (14, 15), more ready utilization through a "transpeptidation" mechanism (16), protection of the amino acid in the peptide from bacterial enzymes which degrade free amino acids (17), and absorption and assimilation by the cell of structures not closely related to those which are affected by the inhibitor (18). Dunn, et al. (19) have shown that inhibitors of phenylalanine utilization do not affect the utilization of a tripeptide with phenylalanine as the central amino acid; however, the utilization of two different dipeptides of phenylalanine and the free amino acid is appreciably affected. They suggested that the tripeptide is assimilated and utilized by different routes than the free amino acid or the two dipeptides. Ifland, et al. (20) suggested that peptides of phenylalanine are not utilized at an essential site of the free phenylalanine. Ball, et al. (21) suggested the same mechanism for the ketoacid of valine. From both investigations it was indicated that an "active form" of the amino acid is formed within the cell from different routes of assimilation.

Experimental

The following lactic acid bacteria were studied:

Lactobacillus plantarum ATCC 8014 (formerly Lactobacillus arabinosus)
Lactobacillus delbrueckii (Lactobacillus acidophilus ATCC 4913)[1]
Lactobacillus brevis ATCC 8287
Lactobacillus casei 7469
Leuconostoc citrovorum (Pediococcus cerevisiae ATCC 8081)[1]
Leuconostoc mesenteroides P-60 (Streptococcus species ATCC 8042)[1]
Streptococcus faecalis ATCC 8043

The basal medium (appendix I) of Henderson and Snell (22), with certain modifications, was used for the study of each microorganism. Nearly all the glucose in the growth medium was replaced by arabinose for the study of L. brevis; also, the concentration of acetate was increased and the concentration of citrate was decreased. Salts B solution was substituted for salts C solution. Folinic acid was supplied in the form of reticulogen and $CaCl_2$ was added when Leuc. citrovorum was studied. In all media the amino acid mixture contained no aspartic acid nor glutamic acid. DL or L-asparagine was added to the media in concentrations of 0.5 mg. per ml. of media to replace aspartic acid. Glycyl-L-glutamic acid was supplied in limiting amounts equal to a "glutamic acid equivalent" concentration of 100 ug. per ml. as was free glutamic acid for purposes of comparison. In all studies the L form of glutamic acid was used.

Racks containing 10 rows of 6 tubes per row were used. One complete series of dose-responses was studied in a single row. The following solutions were used in dispensing the final growth medium: (a) Distilled water was dispensed in amounts of 1.0, 0.8, 0.6, 0.4, 0.2, and 0.0 ml. in tubes 1 through 6, respectively. (B) A solution of glycyl-L-glutamic

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[1]Changes in nomenclature verified in letter dated July 29, 1958, to Dr. R. J. Sirny from the American Type Culture Collection.

acid with a concentration of 100 ug. per ml. was dispensed in amounts of 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml. in tubes 1 through 6. (C) Each tube was brought to a total volume of 2 ml. by adding 1 ml. of medium to each.

The prepared tubes and saline solutions for preparation of inocula were sterilized by autoclaving for 5 minutes at 121°C. After the saline cooled to room temperature, the microorganisms, previously centrifuged from an 18 hour-culture, were suspended in the sterilized solution. Each tube was inoculated with one drop of the appropriate suspension.

The tubes were incubated at 37°C for 65-72 hours, and growth was determined by titration to pH 7.3 with an automatic titrator. Each ml. of approximately 0.05 N KOH added by titration was equal to 100 "counts" on the titrator. For graphic presentation the counts were plotted against the initial concentration of glutamic acid in each tube.

Results and Discussion

The results obtained with each microorganism are shown in Figures 1 through 7; therefore, significant stimulatory and inhibitory effects will be discussed. Significant differences in dose-responses were observed among the different microorganisms; also, differences were noted in the dose-responses of a microorganism at different pH values of 6.0 and 7.0. In all seven bacteria the dose-response to free glutamic acid is greater in the presence of the weaker inhibitor, asparagine, than in the presence of the stronger inhibitor, aspartic acid, at both pH values of 6.0 and 7.0. Varying degrees of dose-responses are observed when the free glutamic acid is replaced with glycyl-L-glutamic acid.

L. plantarum, Leuc. mesenteroides, L. delbrueckii, and S. faecalis

The effect of aspartic acid and asparagine upon the utilization of glutamic and glycyl-L-glutamic acid in *Lactobacillus plantarum*

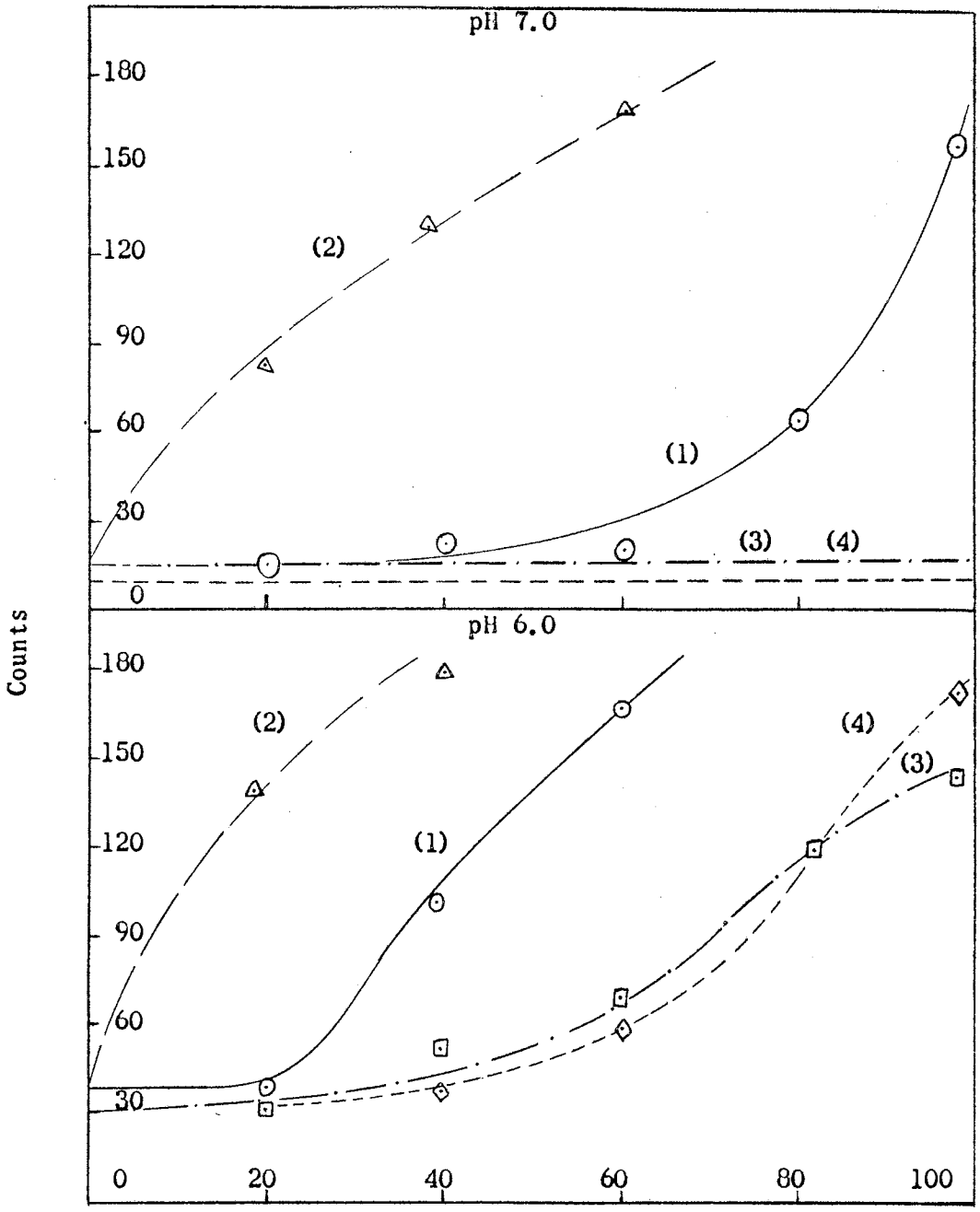

Figure 1. (1) Glutamic acid and 2 mg. aspartic acid (2) Glutamic acid and 0.5 mg. asparagine (3) Glycylglutamic acid and 2 mg. aspartic acid and (4) Glycylglutamic acid and 0.5 mg. asparagine

The effect of aspartic acid and asparagine upon the utilization of glutamic and glycyl-L-glutamic acid in *Leuconostoc mesenteroides*

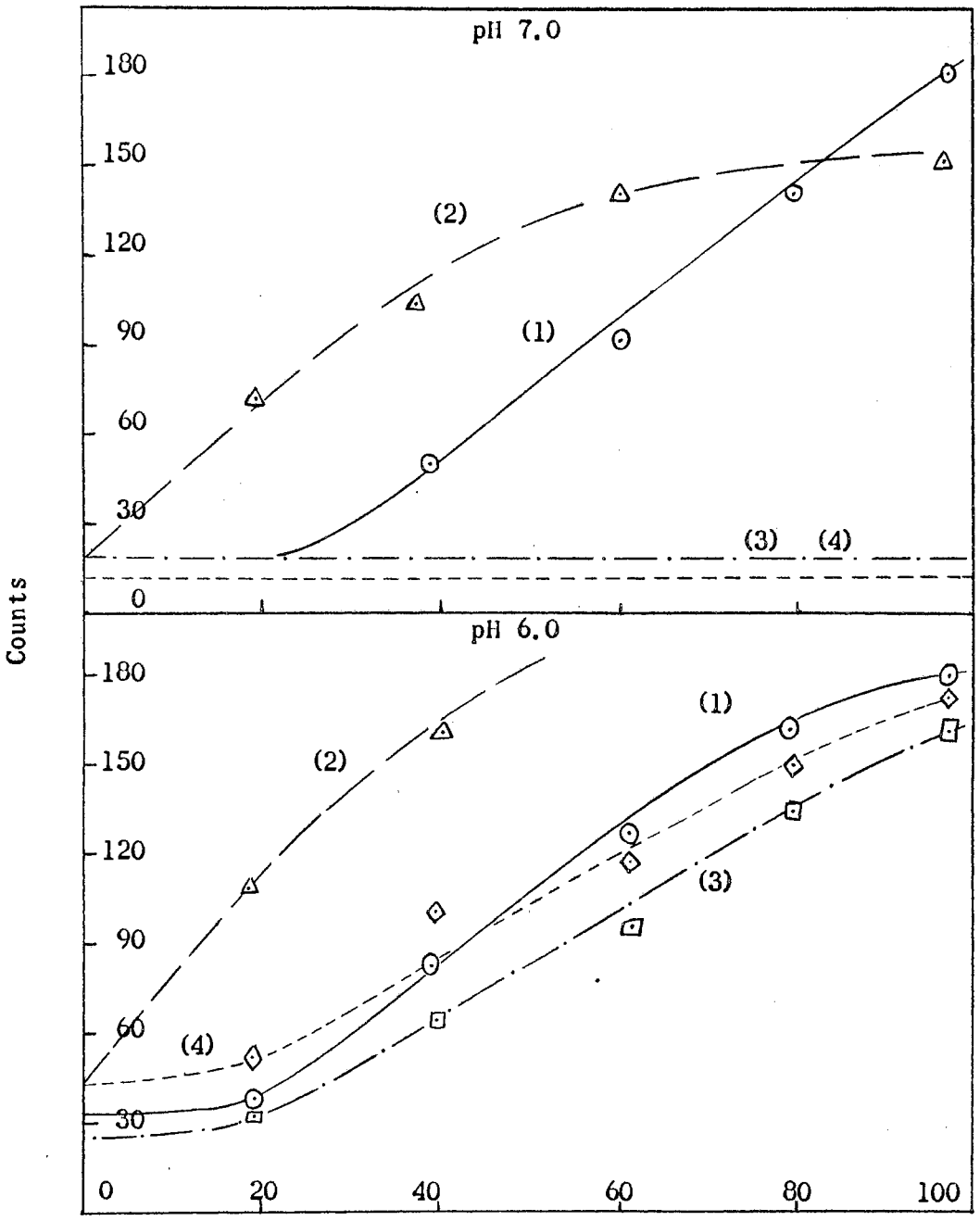

Figure 2. (1) Glutamic acid and 2 mg. aspartic acid and (2) Glutamic acid and 0.5 mg. asparagine (3) Glycylglutamic acid and 2 mg. aspartic acid and (4) Glycylglutamic acid and 0.5 mg. asparagine

The effect of aspartic acid and asparagine upon the utilization of glutamic and glycyl-L-glutamic acid in Lactobacillus brevis

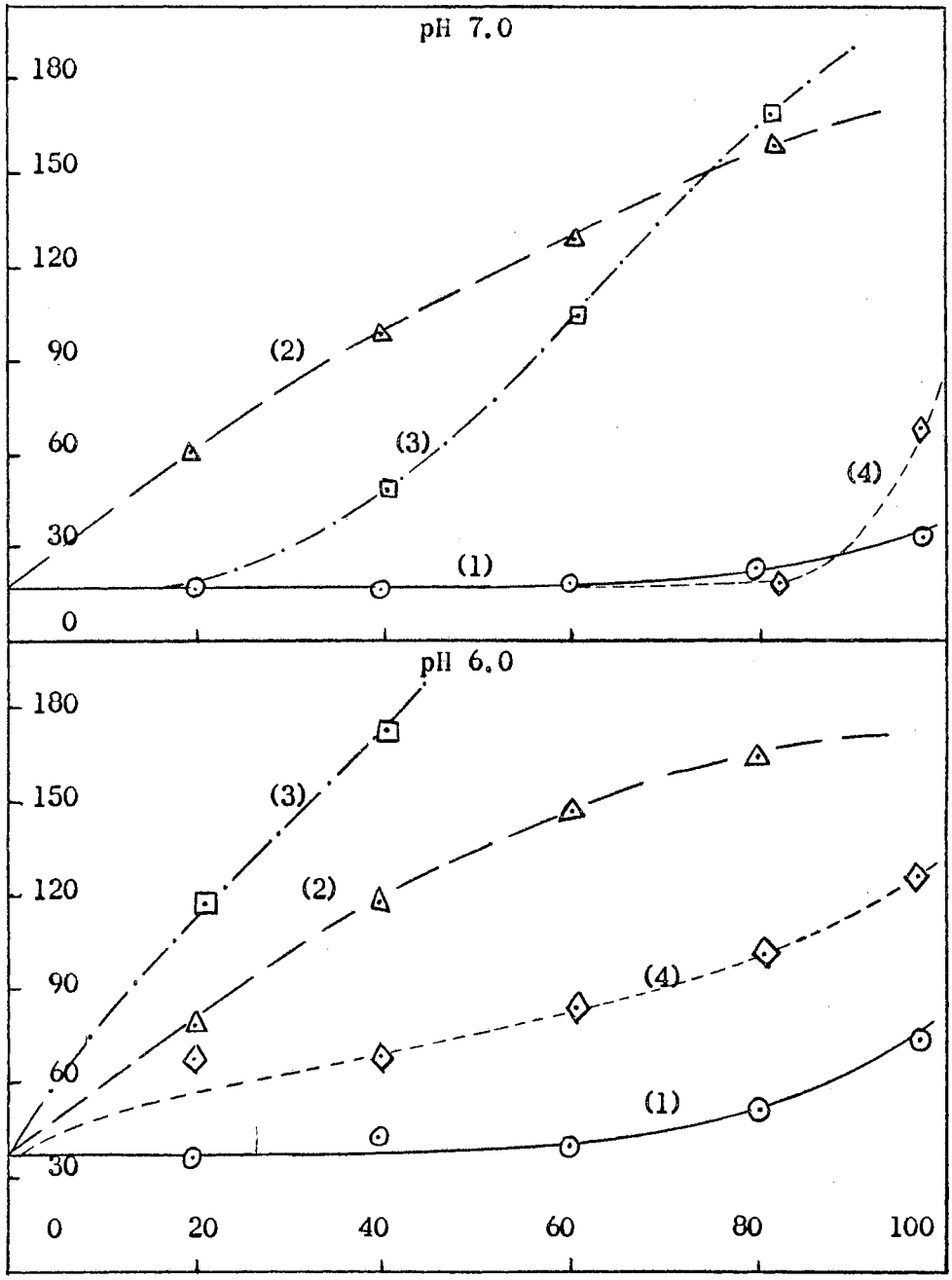

Figure 3. (1) Glutamic acid and 2 mg. aspartic acid (2) Glutamic acid and 0.5 mg. asparagine (3) Glycylglutamic acid and 2 mg. aspartic acid (4) Glycylglutamic acid and 0.5 mg. asparagine

The effect of aspartic acid and asparagine upon the utilization of glutamic and glycyl-L-glutamic acid in *Lactobacillus casei*

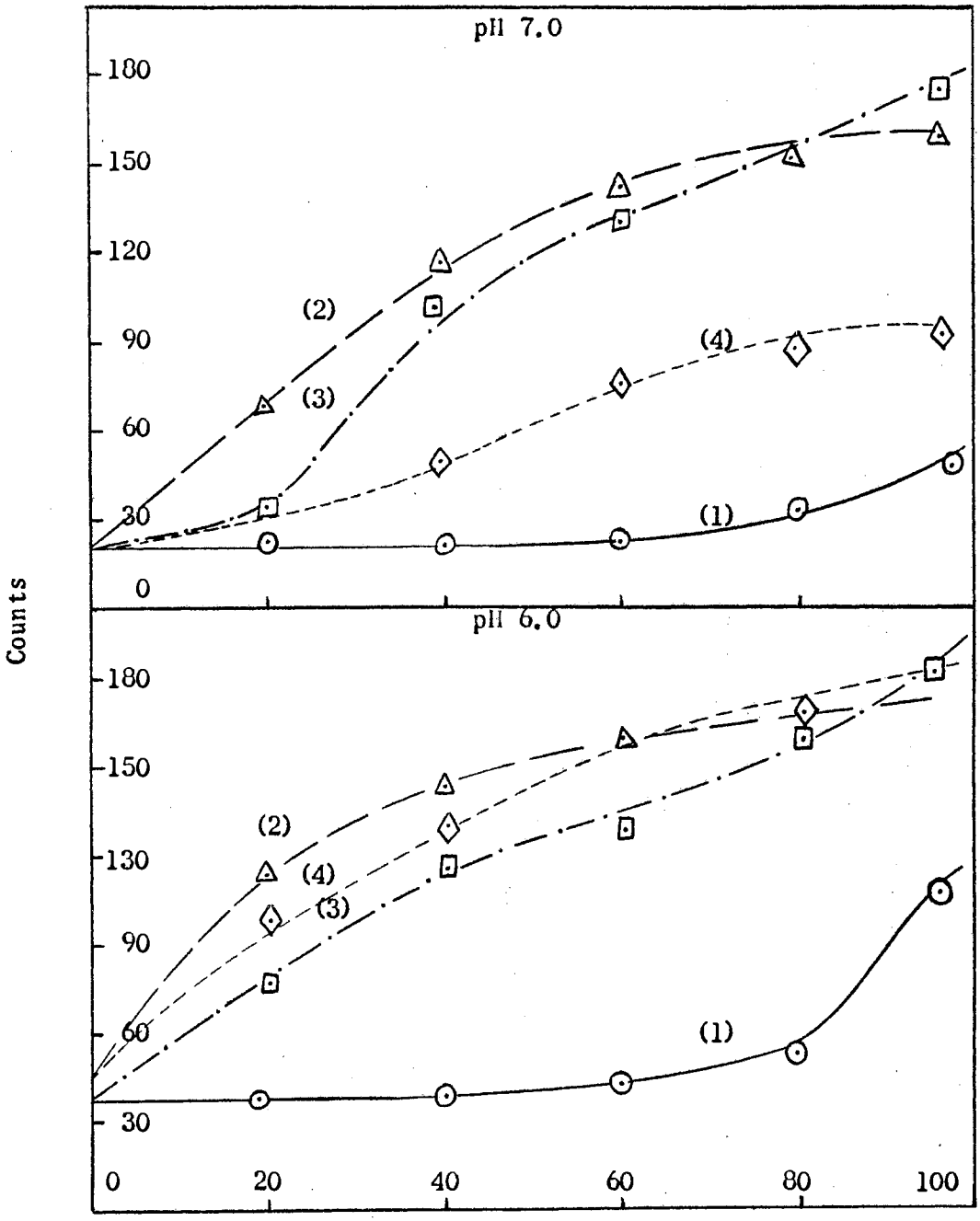

Figure 4. (1) Glutamic acid and 2 mg. aspartic acid (2) Glutamic acid and 0.5 mg. asparagine (3) Glycylglutamic acid and 2 mg. aspartic acid (4) Glycylglutamic acid and 0.5 mg. asparagine

The effect of aspartic acid and asparagine upon the utilization of glutamic and glycyl-L-glutamic acid in Leuconostoc citrovorum

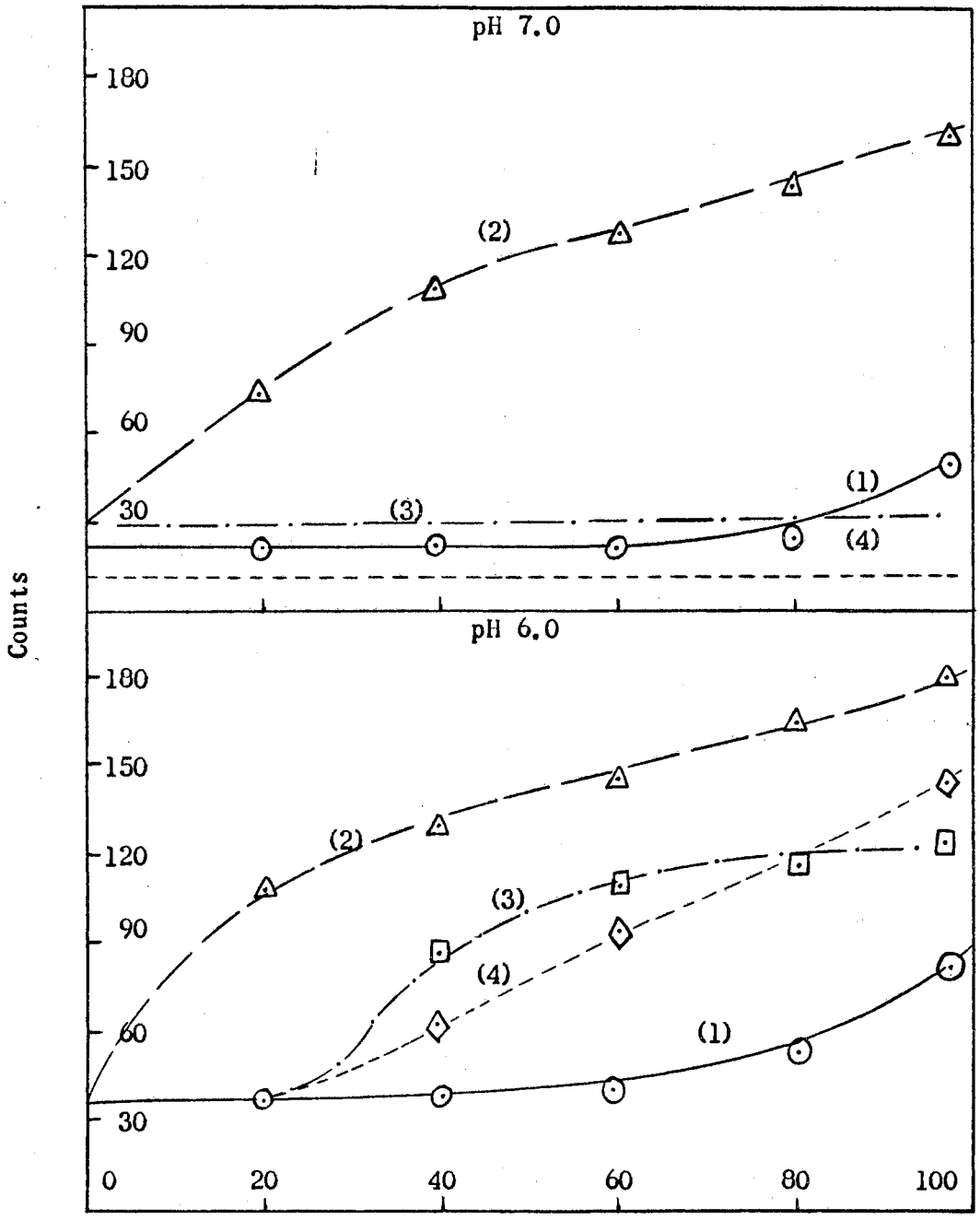

Figure 5. (1) Glutamic acid and 2 mg. aspartic acid (2) Glutamic acid and 0.5 mg. asparagine (3) Glycylglutamic acid and 2 mg. aspartic acid (4) Glycylglutamic acid and 0.5 mg. asparagine

The effect of aspartic acid and asparagine upon the utilization of glutamic and glycyl-L-glutamic acid in Lactobacillus delbrueckii

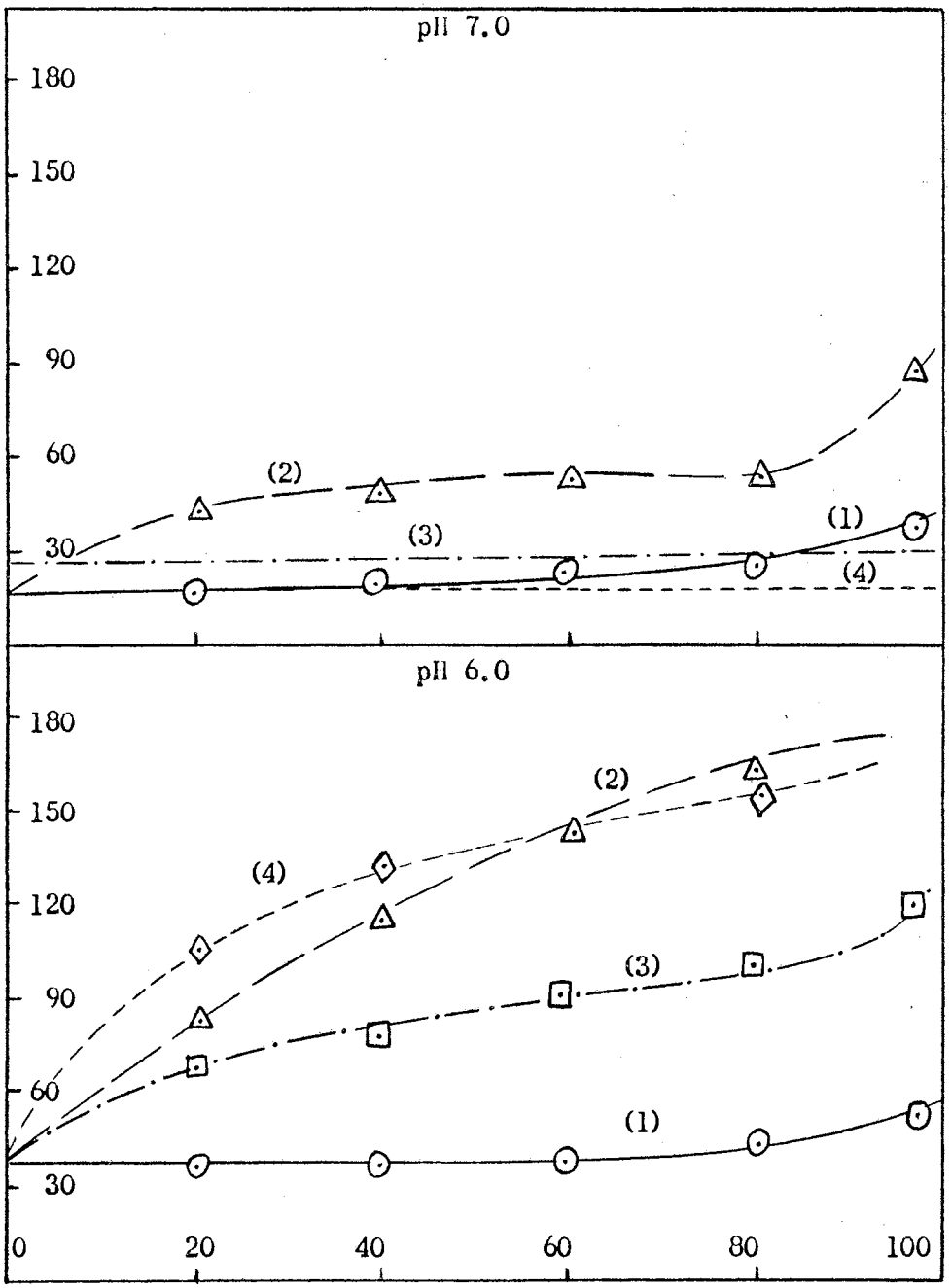

Figure 6. (1) Glutamic acid and 2 mg. aspartic acid (2) Glutamic acid and 0.5 mg. asparagine (3) Glycylglutamic acid and 2 mg. aspartic acid (4) Glycylglutamic acid and 0.5 mg. asparagine

The effect of aspartic acid and asparagine upon the utilization of glutamic and glycyl-L-glutamic acid in Streptococcus faecalis

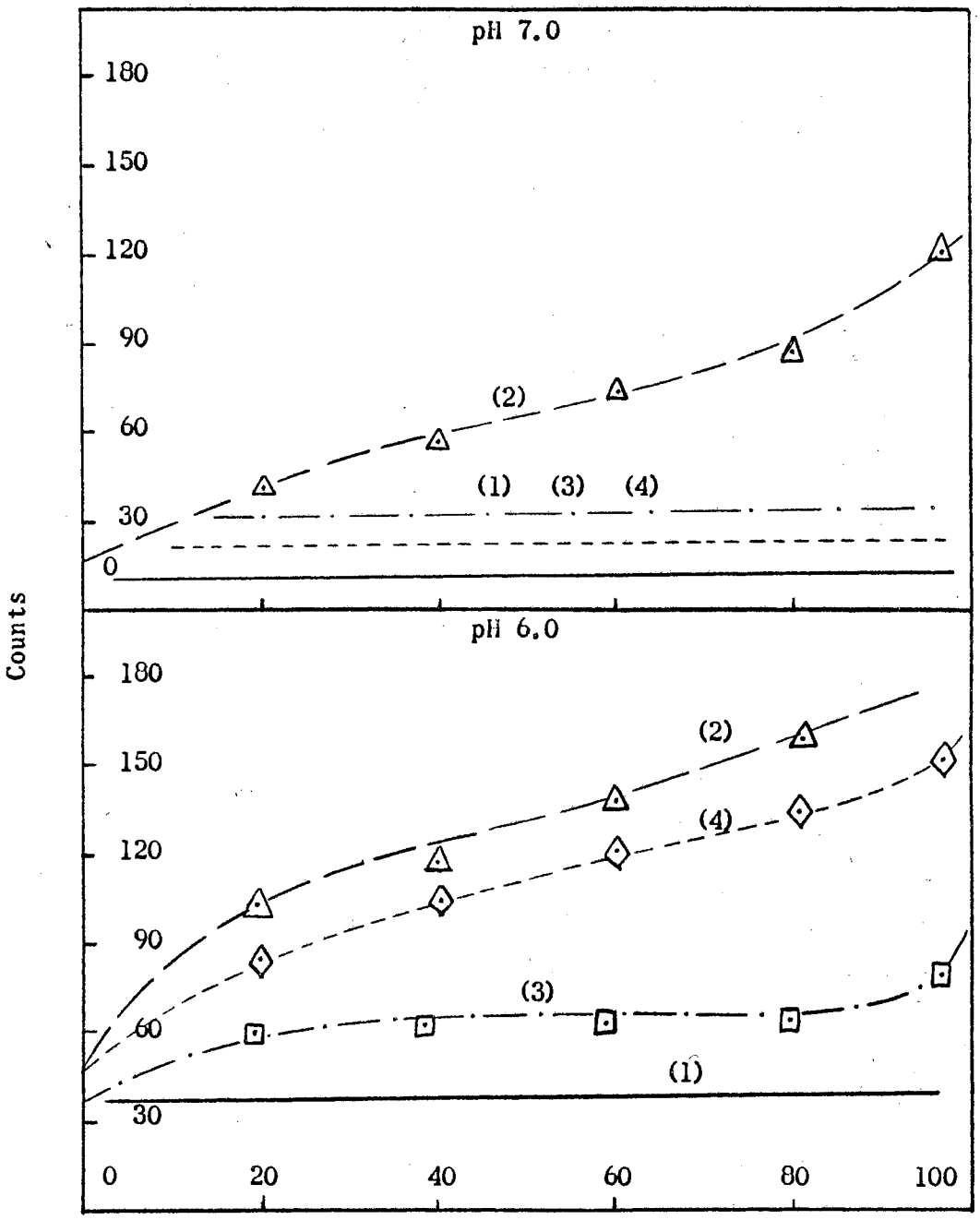

Figure 7. (1) Glutamic acid and 2 mg. aspartic acid (2) Glutamic acid and 0.5 mg. asparagine (3) Glycylglutamic acid and 2 mg. aspartic acid (4) Glycylglutamic acid and 0.5 mg. asparagine

are unable to utilize the peptide at pH 7.0 (Figures 1, 2, 6, and 7). At pH 6.0, however, with L. plantarum (Figure 1) and Leuc. mesenteroides (Figure 2), the peptide is utilized, and essentially equivalent growth is obtained in the presence of both inhibitors. In contrast, L. delbrueckii (Figure 6) utilizes the peptide to a greater extent in the presence of asparagine than in the presence of aspartic acid. It has been previously noted that this latter effect of inhibitors with L. delbrueckii and S. faecalis also applies to the utilization of the free glutamic acid in these two microorganisms. With Leuc. citrovorum (Figure 5) the peptide is utilized only at pH 6.0.

The utilization of the peptide by L. brevis (Figure 3) at pH 7.0 is very slight, but at pH 6.0 a relatively high dose-response is obtained on the peptide form. Furthermore, the dose-response to the peptide is greater than to the free amino acid. This "stimulatory" effect of the peptide is most significant, in that the stronger inhibitor, aspartic acid, almost completely prevents the utilization of the free glutamic acid. With the presence of the weaker inhibitor, asparagine, the dose-response to the peptide is inhibited at pH 6.0 and 7.0 more than by aspartic acid. As previously stated four of the microorganisms are inhibited more by aspartic acid than by asparagine. With L. casei (Figure 4) the peptide is utilized to the same extent as with L. brevis at pH 7.0, but at pH 6.0 aspartic acid inhibits the dose-response to a greater extent than asparagine.

A reduced aspartic acid inhibition of glutamic acid utilization at pH 6.0 and 7.0 is observed in all bacteria except L. plantarum and Leuc. mesenteroides at pH 6.0 and L. delbrueckii (Figure 6) at pH 7.0 by replacing the amino acid with the peptide. At pH 7.0 with L. delbrueckii,

the peptide is utilized less than the amino acid; however, at pH 6.0 the amino acid is utilized more than the peptide. These differences are not observed in L. plantarum and Leuc. mesenteroides. The above results are not as significant as the previous results with L. brevis.

A more extensive investigation of the effect of pH on the dose-response was made with L. brevis (Figure 8). In the same experiment two inocula of different ages were used, 24 hour and 48 hour. It is shown in Figure 8 that more growth occurs with the 24 hour inoculum. Changes in the dose-response are observed with the utilization of the peptide in the presence of aspartic acid. The utilization of the free amino acid (Figure 3) is nearly constant in the presence of aspartic acid at pH 6.0 and 7.0 as compared to the utilization of the peptide-bound amino acid.

The utilization of glutamic acid in glutathione (γ-L-glutamyl-L-cysteinyl-glycine) was compared to that of glycyl-L-glutamic acid with L. brevis, L. casei, and Leuc. citrovorum. The glutathione-bound glutamic acid is not utilized to any extent with L. brevis (Figure 9) or Leuc. citrovorum (Figure 11) as compared to a significant amount of glycyl-L-glutamic acid utilization by the same two microorganisms. L. casei (Figure 10) responds to both peptides of glutamic acid in the presence of aspartic acid or asparagine. The dose-response to glutathione is less than that of glycyl-L-glutamic acid at pH 6.0, but at pH 7.0 the utilization of both is in equal amounts. In these three microorganisms glycyl-L-glutamic acid appears to be utilized better than glutathione.

The effect of (a) age of inoculum and (b) pH on the utilization of glycyl-L-glutamic acid in *Lactobacillus brevis*

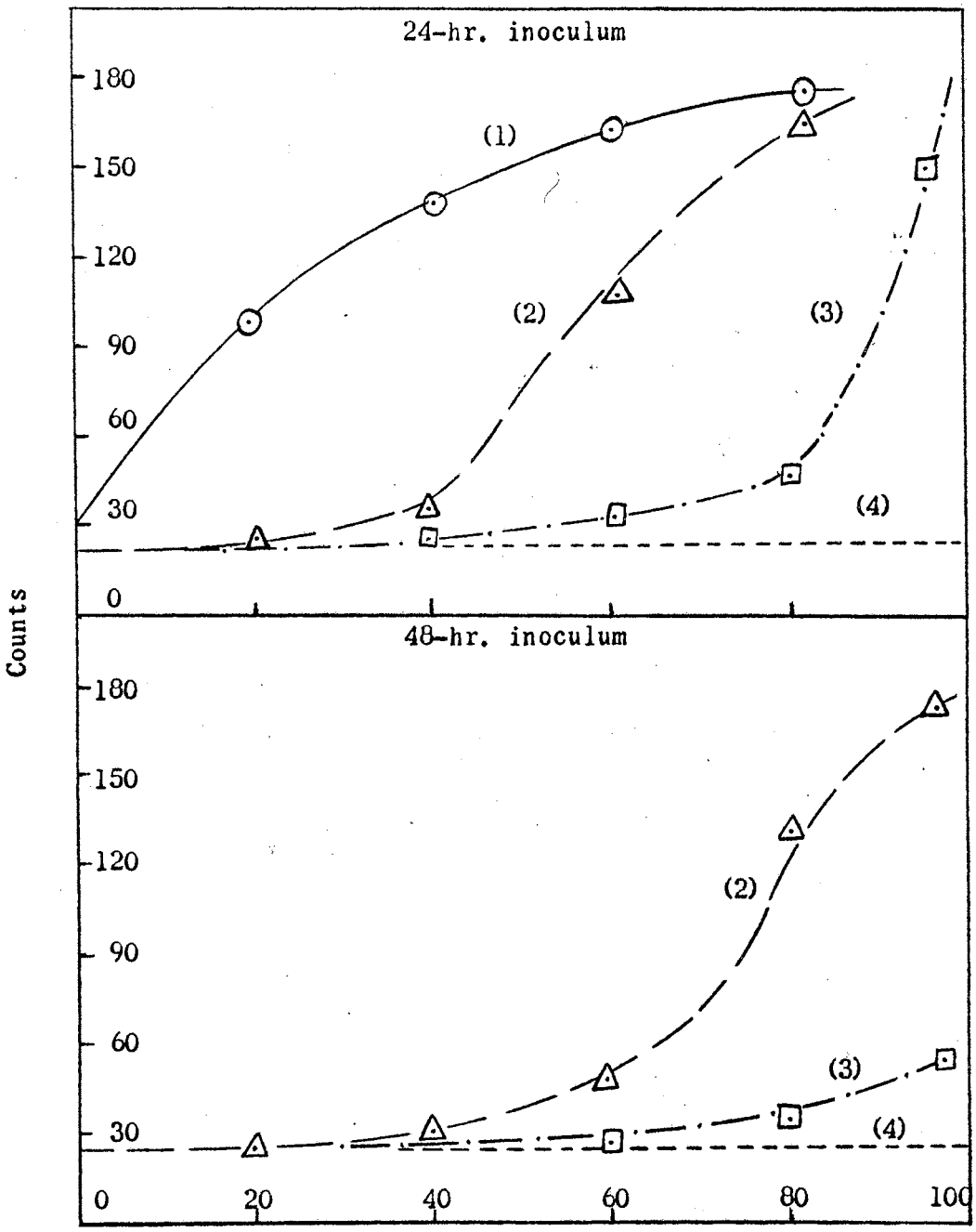

Figure 8. Glycylglutamic acid and 2 mg. aspartic acid (1) pH 6.0 (2) pH 6.7 (3) pH 7.0 (4) pH 7.3

Comparison of the utilization of glycyl-L-glutamic acid and γ-L-glutamyl-L-cysteinyl-glycine in Lactobacillus brevis

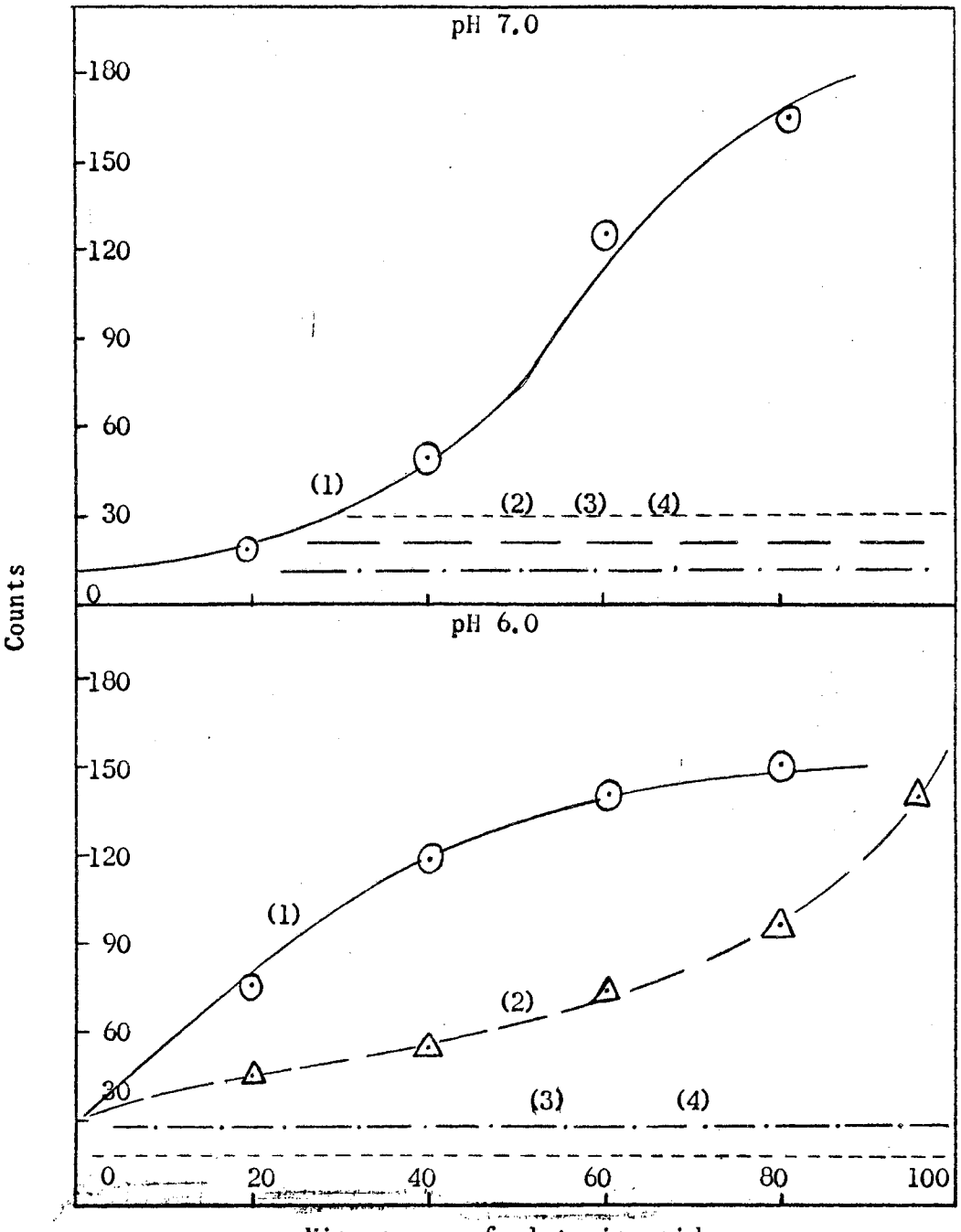

Figure 9. (1) Glycylglutamic acid and 2 mg. aspartic acid (2) Glycylglutamic acid and 0.5 mg. asparagine (3) Glutamylcysteinylglycine and 2 mg. aspartic acid (4) Glutamylcysteinylglycine and 0.5 mg. asparagine

Comparison of the utilization of glycyl-L-glutamic acid and γ-L-glutamyl-L-cysteinyl-glycine in Lactobacillus casei

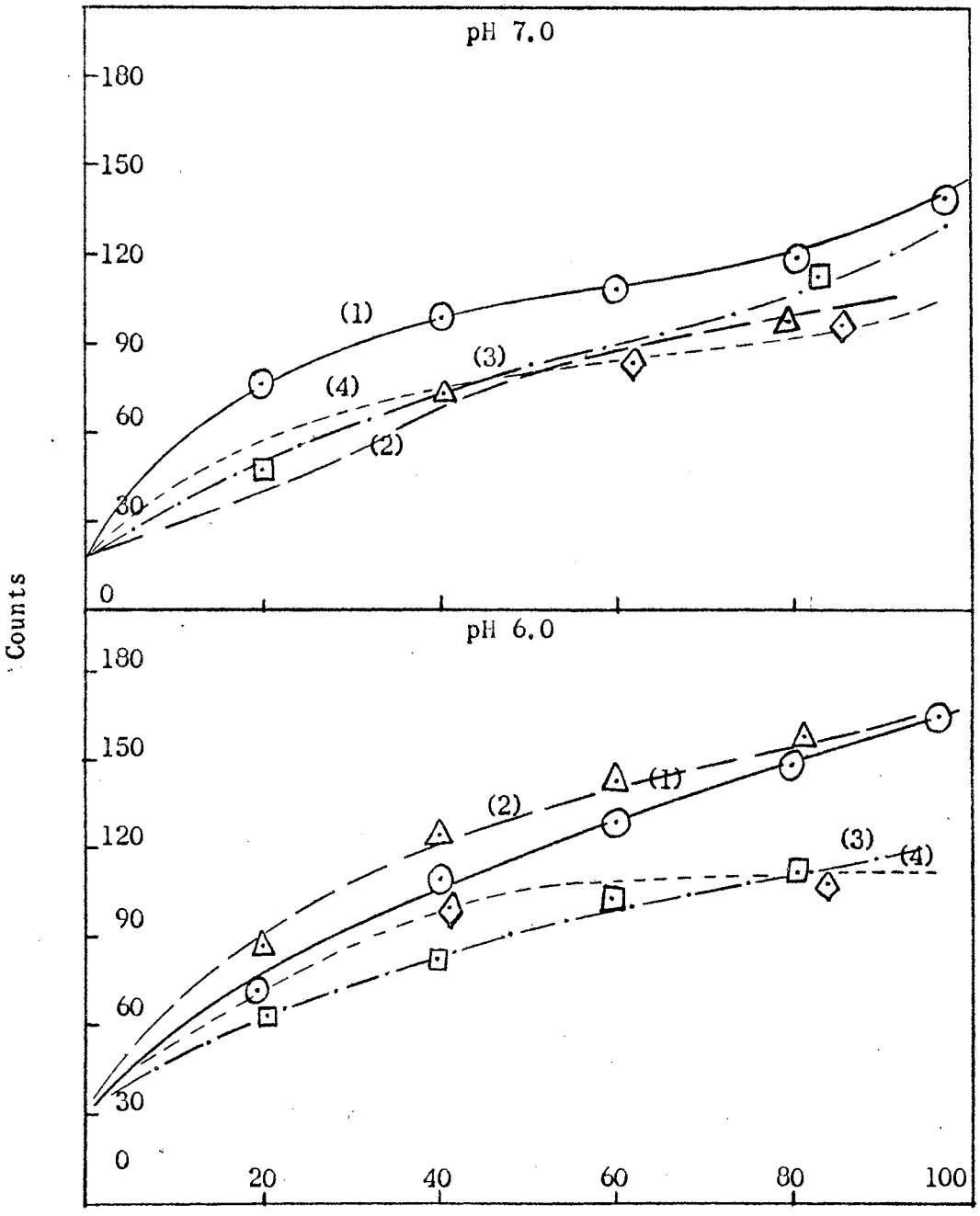

Figure 10. (1) Glycylglutamic acid and 2 mg. aspartic acid (2) Glycylglutamic acid and 0.5 mg. asparagine (3) Glutamylcysteinylglycine and 2 mg. aspartic acid (4) Glutamylcysteinylglycine and 0.5 mg. asparagine

Comparison of the utilization of glycyl-L-glutamic acid and γ-L-glutamyl-L-cysteinyl-glycine in *Leuconostoc citrovorum*

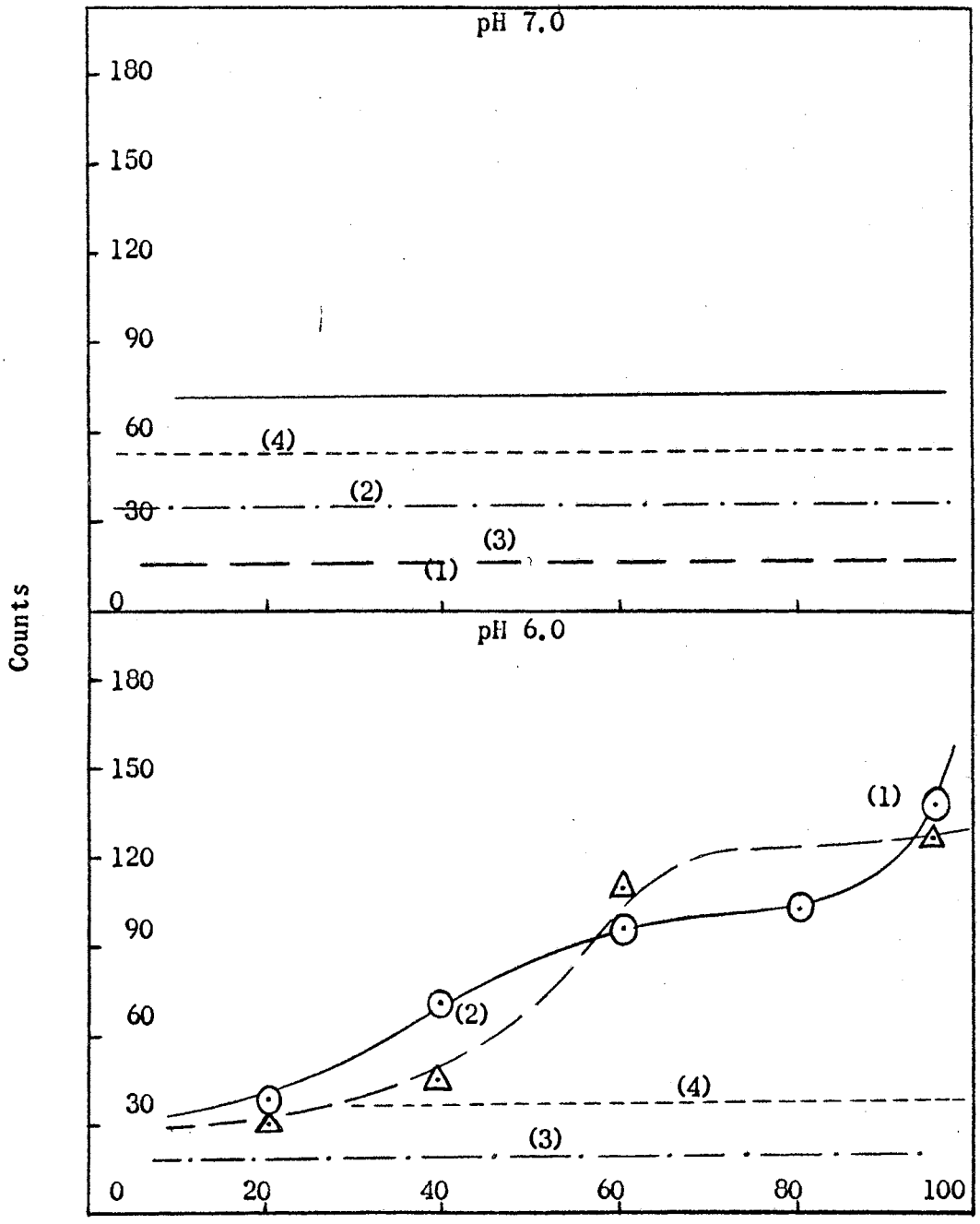

Figure 11. (1) Glycylglutamic acid and 2 mg. aspartic acid (2) Glycylglutamic acid and 0.5 asparagine (3) Glutamyl-cysteinylglycine and 2 mg. aspartic acid (4) Glutamyl-cysteinylglycine and 0.5 mg. asparagine

Summary

A survey of seven lactic acid bacteria showed that their growth-responses were generally greater with free glutamic acid than with peptide-bound glutamic acid. The inhibition of free glutamic acid utilization was always greater in the presence of aspartic acid than asparagine at pH values 6.0 and 7.0. Various degrees of peptide-bound glutamic acid utilization were observed at pH values 6.0 and 7.0 and in the presence of aspartic acid and asparagine. A striking peptide "stimulation" was observed with L. brevis at pH 6.0 in the presence of aspartic acid which inhibits almost completely the utilization of free glutamic acid under identical conditions.

CHAPTER III

STUDIES OF GLUTAMIC ACID UPTAKE IN *LACTOBACILLUS PLANTARUM*

Introduction

The purpose of studying the uptake of glutamic acid by cells of *L. plantarum* was to determine whether or not the aspartic acid inhibition of the utilization of glutamic acid could be explained by the entry of glutamic acid through the cell wall or membrane. Previous studies have suggested that the cell wall is a possible site of the antagonisms between amino acids and their analogs. For example, Holden (23) showed that growth of *S. faecalis* and *L. plantarum* was inhibited by phenylpyruvate in a tyrosine-deficient medium and antagonized by para-hydroxyphenylpyruvate in a phenylalanine-deficient medium. As an explanation he suggested the inhibition of growth might be due to either inhibition of enzyme systems or interference with cell entry.

Amino acids can enter a bacterial cell either by simple diffusion or by an active transport system; for the latter an external source of energy is required. Gale (24) has contributed much work to the study of the passage of glutamic acid and other amino acids into bacterial cells. Riggs, et al. (25) have extensively studied the mechanism of the active transport system of amino acids in Ehrlich mouse ascites carcinoma cells. Rowlands et al. (26) investigated the uptake of glutamic acid peptides by bacterial cells and compared that to the uptake of free glutamic acid.

There has been no previous attempt in the literature to explain the aspartic acid inhibition of the utilization of glutamic acid on the basis of entry at the cell wall as indicated by direct experimental evidence. In this study a correlation was formulated between the uptake of glutamic acid into the bacterial cell and the growth of the cell, both under similar conditions.

Experimental

Preparation of Resting Cells

The cells were transferred from stab cultures, prepared fresh every two weeks, to a 2-ml. broth culture and incubated at 37°C for 15 hours. A 1-liter volume of modified Henderson-Snell media (Appendix I) was prepared with a limiting amount of glutamic acid present (50 micrograms/ml.). The cells were centrifuged, transferred to the 1-liter of medium, and incubated at 37°C for 18-20 hours. The cells were centrifuged, washed with Gale's solution, and resuspended in 25 ml. of Gale's solution (Appendix II).

Reagents

All reagents were dissolved in Gale's solution. Stock solutions were prepared and stored in the refrigerator under toluene; exact concentrations were determined by micro-biological assay procedures. Solutions were prepared of glucose, glutamic acid, aspartic acid, and asparagine.

Procedure

Each experiment was performed in a 22 by 180 mm. glass test tube. Glucose and the amino acids to be studied were added to each tube. The

tubes were incubated at 37°C for 30 minutes in order to obtain a constant temperature.

At zero time a 4-ml. suspension of resting cells was added to each tube, bringing the final volume to 10 ml. The initial concentration of glutamic acid was 10 micromoles per ml. and aspartic acid and asparagine were 50 micromoles per ml. At regular time intervals an aliquot (1.1-1.5 ml.) was taken, centrifuged for 7 minutes at approximately 1000 r.p.m. and exactly 1.0 ml. of the supernatant was taken for analysis.

Each 1-ml. aliquot was autoclaved for 5 minutes in order to destroy any contamination and analyzed for glutamic acid by a standard microbiological assay procedure. The amount of glutamic acid which disappeared from the external medium was assumed to be taken up by the resting cells.

To clearly portray the results, the amount of glutamic acid taken up by the resting cells was plotted against time. Graphs were prepared from each experiment in the same manner, so comparisons could be made under different experimental conditions. The pH (7.0), temperature (37°), and concentrations of all solutions were initially constant.

Results and Discussion

The uptake of glutamic acid by resting cells of Lactobacillus plantarum was investigated under inhibitory conditions in the presence of aspartic acid and asparagine. Figures 12-14 show the effect of these amino acids upon the uptake of glutamic acid.

The amount of glutamic acid taken up by the cells is expressed on the basis of 100 mg. dry weight of cells. When other amino acids are present in the external medium, the rate of glutamic acid uptake is

Glutamic acid uptake by resting cells
of Lactobacillus plantarum

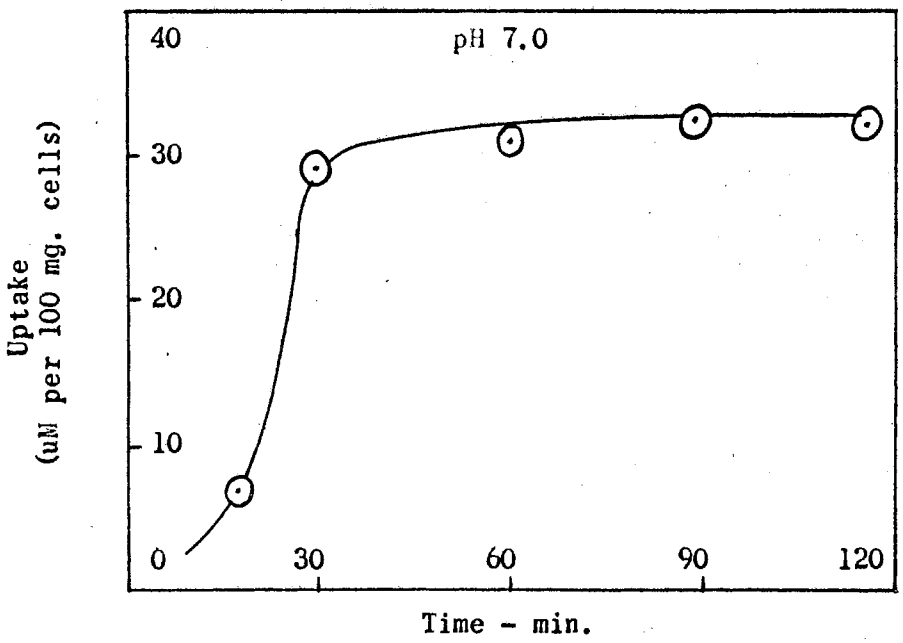

Figure 12. Contents of medium: glucose 10 micromoles/ml., glutamic acid 10 micromoles/ml., and cell conc. 6.4 mg./ml.: total vol. = 10 ml.

Glutamic acid uptake by resting cells
of Lactobacillus plantarum

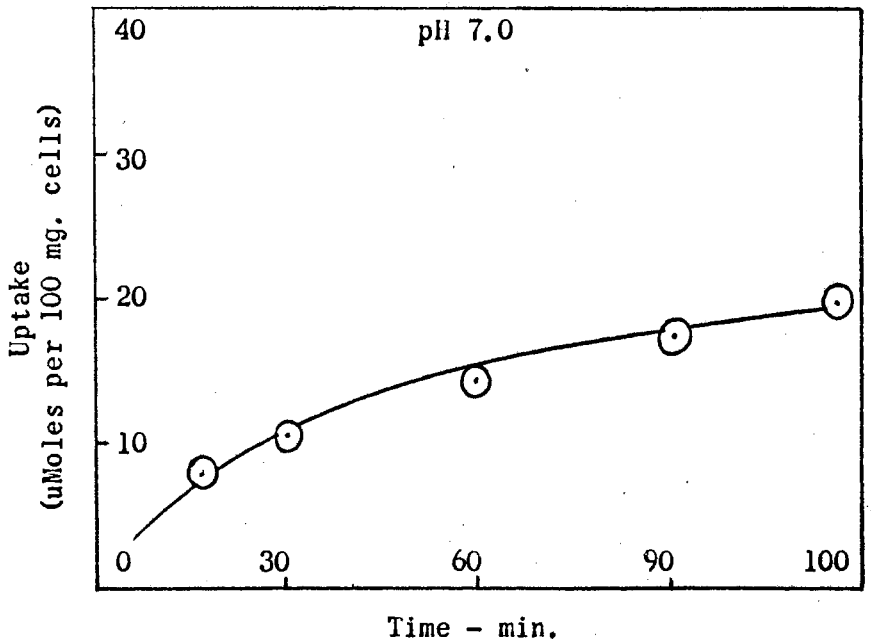

Figure 13. Contents of medium: glucose, 10 micromoles;ml., glutamic acid 10 micromoles/ml., asparagine 50 micromoles/ml., and cell conc. 6.4 mg./ml.: total vol. = 10 ml.

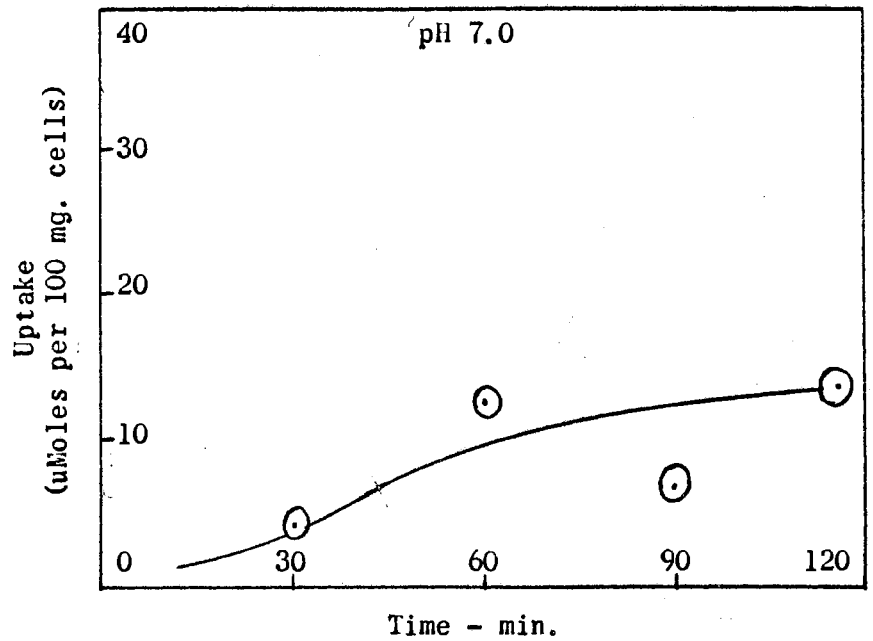

Figure 14. Contents of medium: glucose 10 micromoles/ml., glutamic acid 10 micromoles/ml., aspartic acid 50 micromoles/ml., and cell conc. 6.4 mg./ml.: total volume = 10 ml.

decreased as well as the total amount of uptake.

The degree of the decrease in glutamic acid uptake in the presence of aspartic acid or asparagine can be correlated with the lag phase of the growth curve under similar conditions. Figures 15-17 show the effect of inhibitory concentrations of asparagine and aspartic acid upon growth, which was followed by turbidimetric measurements on a Coleman Nephelometer.

In Figure 15, the lag phase increases with an initially smaller amount of glutamic acid present in the external medium, until no growth occurs at lower concentrations. In Figure 17, the replacement of aspartic acid with asparagine eliminates the lag phase, or rather, the lag phase is shortened sufficiently so that it cannot be measured accurately under the conditions employed. The appearance and disappearance of the lag phase can be explained in terms of the curves in Figures 12 through 14 showing the uptake of glutamic acid; however, it should be noted that the lag phases are determined in a medium of 16 amino acids in addition to glutamic acid and asparagine or aspartic acid. Figure 16 represents a dose-response curve which was derived from the final values in Figures 15 and 17. The curves of the uptake of glutamic acid are related to these curves in the same manner as to the time-growth curves.

In the discussion of the above results, it can be stated that entry of glutamic acid at the cell wall or membrane contributes a significant amount to the lag phase of the normal time-growth curve and to the dose-response curve of L. plantarum. The relationship between the uptake curves and the growth curves varies depending on how one interprets the results. Only two amino acids were "competing" for entry into the cell during the uptake study, whereas growth was determined while 18 amino

The response of Lactobacillus plantarum to glutamic acid in a time-growth study

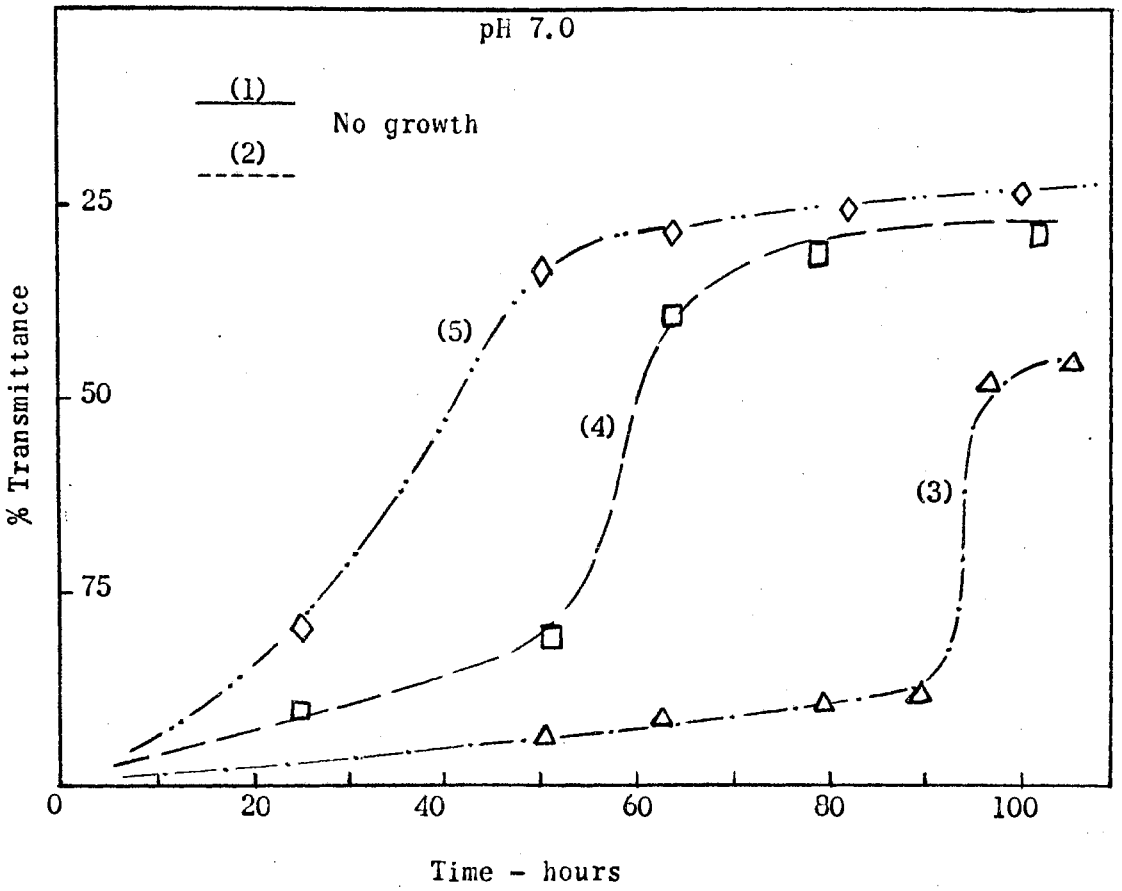

Figure 15. Each tube contains 2 ml. total volume with 2 mg. aspartic acid present. Glutamic acid conc. = (1) 20 (2) 40 (3) 60 (4) 80 and (5) 100 micrograms per 2 ml.

A turbidimetric measurement of the dose-response of
Lactobacillus plantarum to glutamic acid

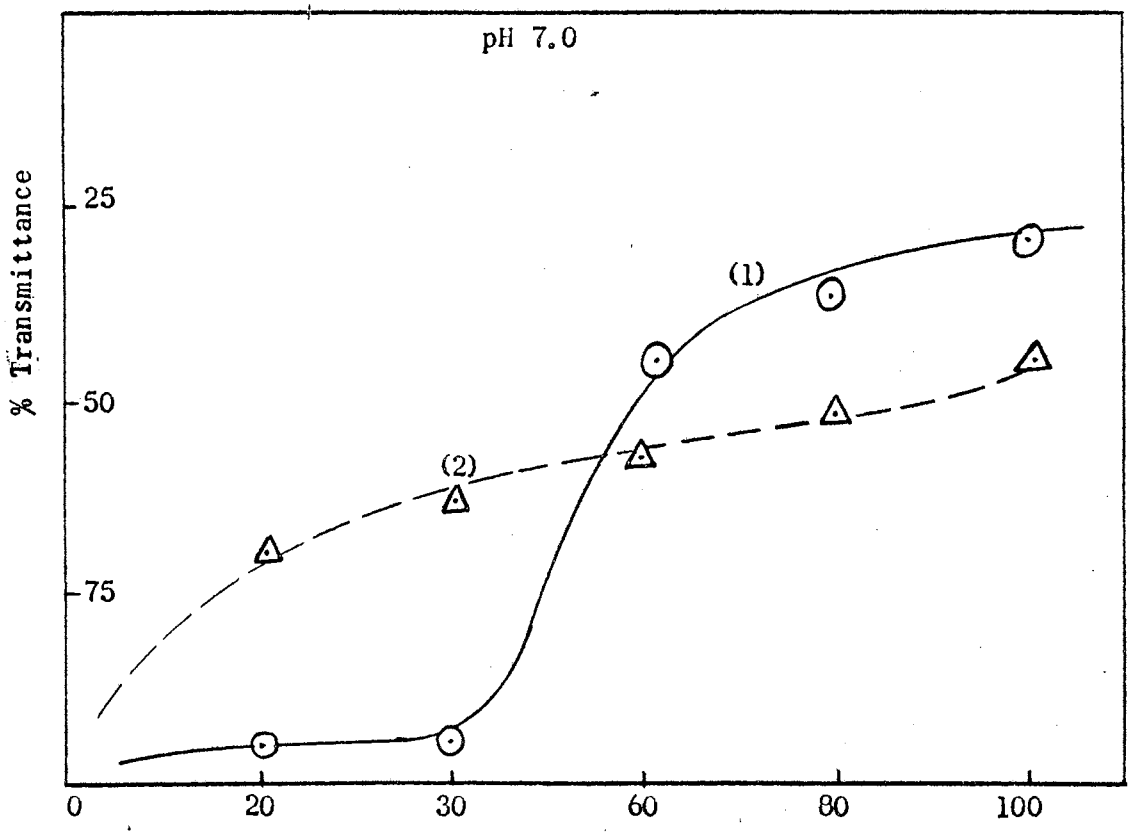

Figure 16. Curve 1 = aspartic acid, curve 2 = asparagine

The utilization of glutamic acid by
Lactobacillus plantarum

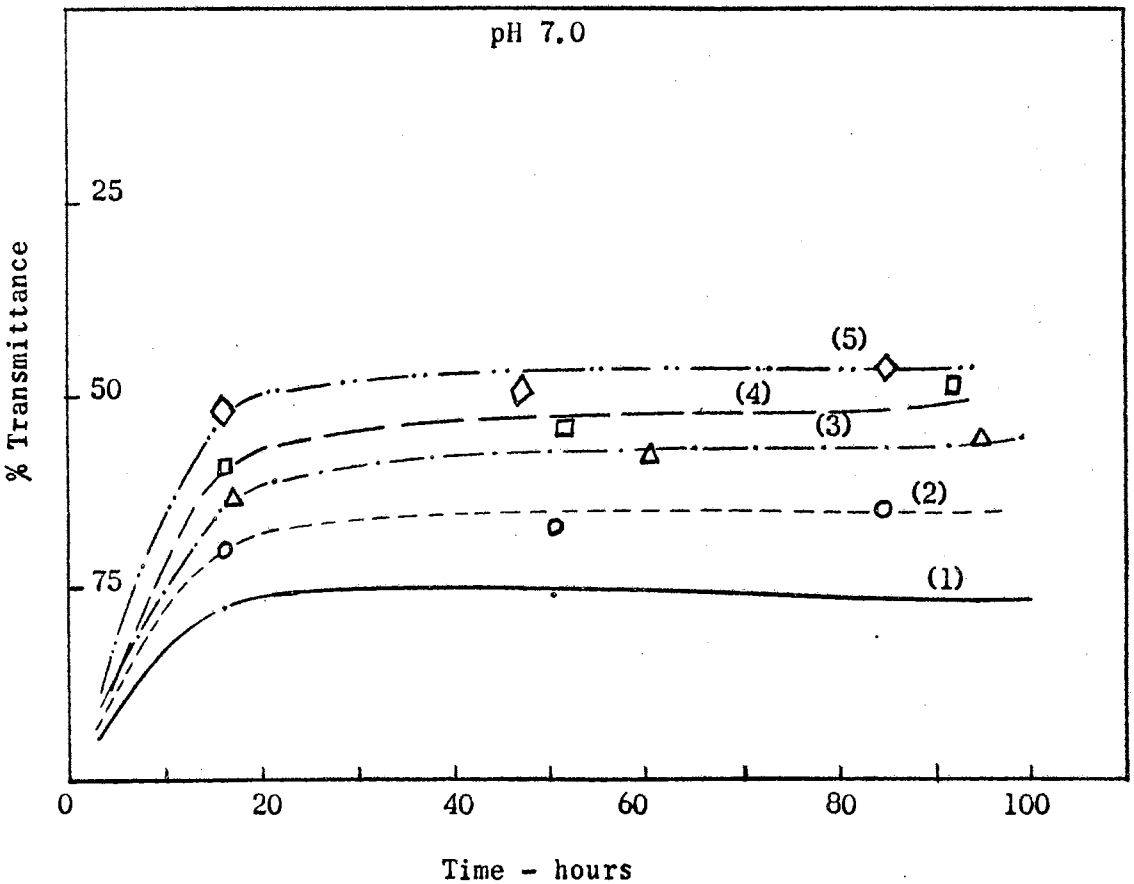

Figure 17. Each tube contains 2 ml. total volume with 2 mg. asparagine present. Glutamic acid conc. = (1) 20 (2) 40 (3) 60 (4) 80 and (5) 100 micrograms per 2 ml.

acids were "competing" for entry into the cell. These observations do not give any clues as to the mechanism of an amino acid competition at the cell wall. It seems that until the mechanism of amino acid transport across cell walls and membranes is definitely revealed, the mechanism of amino acid competition at the cell wall cannot be fully explained.

Speculation as to possible mechanisms of amino acid uptake other than those previously mentioned suggests many approaches for future study. The descriptive chemistry will not answer the questions of why or how. There might be specific "uptake" enzymes involved; perhaps there is an enzyme for each amino acid. Intermediate products may be formed; if so, they are probably found in very minute quantities which suggests more sensitive quantitative techniques.

For glutamic acid, it appears that measurement of disappearance from the external medium as a criterion for measurement of the uptake of the amino acid is a sound procedure; however, the microbiological assay for glutamic acid has specific limitations under the conditions involved. There is difficulty in assaying for glutamic acid in the presence of an excess amount of aspartic acid. Another limitation is that the microbiological assay depends upon the ability of living organisms to yield reproducible results: living organisms are sometimes very responsive to small changes in their environmental conditions. Technical difficulties are usually encountered when established conditions must be varied. These difficulties can usually be worked out in time, but other techniques merit consideration. A possibility for the assay of glutamic acid is the specific glutamic acid decarboxylase which has been used so extensively by other investigators. The use of C^{14}-labeled glutamic acid or tritium-labeled glutamic acid peptides can possibly provide a more accurate and

more direct method of quantitative assay. Radioactive amino acids and peptides seem to be very promising tools for future research on amino acid uptake.

The growth responses of the lactic acid bacteria differ when glutamic acid is available to the microorganism in free form or in peptide-bound form. Significant differences are noted among different bacteria under the same environmental conditions. From the results of the survey, a logical organism for future study is Lactobacillus brevis. The dose-responses of this organism are very high with the peptide-bound glutamic acid and very low with free glutamic acid. A study of the uptake of the free and peptide-bound amino acid by Lactobacillus brevis seems to be a most promising study.

This investigation shows various differences in the dose-responses of the specific organisms to free and peptide-bound glutamic acid. Similar differences in the uptake of free and peptide-bound amino acids may be expected among different species of organisms. Results of amino acid assimilation by specific organisms and their responses to amino acids and peptides can be expected to follow a general pattern. It can further be expected that the components of this general pattern will be revealed by studies of these differences encountered in the various organisms.

Summary

Uptake of free glutamic acid by resting cells of L. plantarum was determined, and the results were compared to growth responses of the same organism to glutamic acid when inhibitory concentrations of aspartic acid and asparagine were present in the external medium. At pH 7.0, glutamic acid was taken up in the following amounts per 100 mg. dry

weight of cells: glutamic acid alone, 35 micromoles; glutamic acid and asparagine, approximately 20 micromoles; glutamic acid and aspartic acid, approximately 10 micromoles. The lag phases of L. plantarum under different inhibitory conditions were partially explained from the amount of glutamic acid which was taken up by the cell under similar conditions.

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A P P E N D I X

APPENDIX

I. Basal medium for growth of microorganisms

A. Constituents of basal medium (total volume, 100 ml.)

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 (or B) soln.	4.0 ml.
AGU-soln.	2.0 ml.
X-soln.	2.0 ml.
Vitamin soln.	2.0 ml.
Amino acid soln.	25.0 ml.
Asparagine	50.0 mg.

B. Composition of above constituents

Amino acid composition (glutamic and aspartic acid free)

DL-Alanine	200 mg.	DL-Tryptophan	40 mg.
L-Arginine HCl	40 mg.	DL-Valine	40 mg.
DL-Isoleucine	40 mg.	Glycine	20 mg.
L-Lysine HCl	40 mg.	L-Cystine	20 mg.
DL-Methionine	40 mg.	L-Histidine HCl	40 mg.
DL-Phenylalanine	40 mg.	L-Leucine	20 mg.
DL-Serine	40 mg.	L-Proline	20 mg.
DL-Threonine	40 mg.	L-Tyrosine	20 mg.

Salts C		Salts B	
$FeSO_4$ $7H_2O$	0.5 gm.	$FeSO_4$ $7H_2O$	5.0 gm.
$MnSO_4$ $4H_2O$	2.0 gm.	$MnSO_4$ $4H_2O$	0.25 gm.
$MgSO_4$ $7H_2O$	10.0 gm.	$MgSO_4$ $7H_2O$	0.25 gm.
Dissolve with the aid of HCl to 250 m..		Dissolve with the aid of HCl to 250 ml.	

AGU-soln.		X-soln.	
Adenine-sulfate	250 mg.	Xanthine	250 mg.
Guanine HCl	250 mg.		
Uracil	250 mg.	Dissolved in dilute KOH and made to 250 ml.	
Dissolved with HCl and made to 250 ml.			

Vitamin soln. (total volume, 250 ml.)

Thiamin	25 mg.
Niacin	25 mg.
Ca-pantothenate	25 mg.
Pyridoxal	5.0 mg.
Riboflavin	25 mg.
PABA	5.0 mg.
Biotin*	0.25 mg.
Folic acid**	0.25 mg.

II. Medium for uptake studies (total volume, 10 ml.)

KH_2PO_4	10 mg.
Na_2HPO_4	33 mg.
NaCl	10 mg.
$MgSO_4$ $7H_2O$	7 mg.
Glucose	18 mg.
Appropriate amino acids:	
glutamic acid	14.7 mg.
aspartic acid	66.5 mg.
asparagine	66.0 mg.
Cells (dry weight) Approx.	60.0 mg.

*Biotin stored in soln. in 50% EtOH.

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

VITAE

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Master of Science

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