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

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CHAPTER I

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

Introduction

An important biochemical objective in the field of protein chemistry is the understanding of the process of protein biosynthesis. Studies of amino acid interrelationships and peptide utilization in bacteria have contributed much to our knowledge of nutrition. Such studies are useful in elucidating the pathways of protein biosynthesis and may eventually lead to the formulation of the mechanisms involved in the building of protein molecules *in vivo*.

Many of the recent studies conducted with various microorganisms have indicated many different interrelationships between various nutrients in the microbiological media. A rather large number of these interrelationships have involved the amino acids. For the most part, the studies have involved determination of the effects of varying concentrations of one amino acid on the utilization of another. This has led to a recognition of innumerable examples of paired interrelationships in which only two amino acids have been involved and in which the effects of other amino acids in the medium were not considered. From studies such as these, no general pattern has emerged to adequately explain diverse effects which have been observed. It is, therefore, desirable to

briefly discuss some of the major types of amino acid interrelationships which have been found in bacteria.

Perhaps the most well known type of relationship among amino acids is the type which is termed competitive antagonism. This can be exemplified by the inhibition of the utilization of glutamic acid by aspartic acid and vice versa. At growth-limiting amounts of one, the other inhibits growth presumably because of structural similarity between the two amino acids and consequent competition between the two for reactive sites on the surface of the enzymes involved.

One other type of interrelationship essentially opposite to that of the competitive type was found by Sirny (1) between arginine and proline. In the metabolism of Leuconostoc mesenteroides P-60, a high amount of one amino acid is required for optimal utilization of limiting low amounts of the other. In a later survey made by Wold (2), it was revealed that there are other pairs of amino acids exhibiting the same type of interrelationships. Unlike the competitive type of interdependence, an explanation for the arginine-proline type is not as clear. One possible explanation is that the interrelated amino acids might be involved together in the formation of a compound, possibly a peptide, which serves as precursor in protein synthesis.

In the studies of amino acid interrelationships, it was found that where the utilization of an amino acid in the bacterial medium would be inhibited in any way, peptide source of the amino acid exhibited stimulatory effect. Interrelationship studies have thus led to the wide field of investigation of peptide effect in the metabolism of microorganisms.

Conditions of inhibitions brought about by the imbalance of amino acids in the medium had been employed in the study of the utilization

of peptides and partial hydrolysates by microorganisms. Such investigations attempt to reveal the mechanism of peptide utilization. Where the peptide source is less readily utilized than the free growth-limiting amino acid, it is a highly probable supposition that hydrolysis of the peptide bond must first occur before the peptide source is utilized. On the other hand, direct incorporation of peptides would probably explain their stimulatory effect. The results of many workers, however, do not favor either one of these two hypotheses, but rather indicate that in some cases peptides were utilized directly and in others indirectly, depending upon the type of organism, the specific peptide, and the conditions employed.

In the metabolism of Leuc. mesenteroides, partial hydrolysates, used as peptide sources of proline in the study of the arginine-proline interrelationship, exhibited greater growth promoting activity than did the free proline under certain specific, sub-optimal conditions (1).

The enhanced activity of peptides, in comparison with their constituent amino acids, has now been observed with a variety of antagonistic amino acid pairs and with several different organisms (3-18).

In the investigations of Ravel and Shive (16), arginine was found to inhibit the utilization of either glutamic acid or glutamine in Streptococcus lactis. Glutamine was found to be 500 - 1000 times more effective than glutamic acid in preventing the inhibition. They suggested that arginine competitively prevents the utilization of glutamic acid for biosynthesis more effectively than glutamine. Although glutamine is not a true peptide, it possesses structural similarity to the peptide linkage and might possibly have some of the properties of peptides.

In the inhibition studies of Ifland et al. (5), aspartic acid was

slightly more effective than glycylasparagine in promoting enzyme synthesis in the absence of inhibitor, but less effective in the presence of moderate concentrations of the aspartic acid antagonists. They had proposed that the peptide is utilized by a route not involving the free amino acid, but probably involving a common active intermediate.

In the studies made by Dunn and coworkers (3), peptide sources of limiting amino acid were preferred over the free amino acid. The response of Lactobacillus casei to partial hydrolysates of protein was found to be more utilized than the free amino acid itself. In a later study (10), several dipeptides were tested for activity with several organisms. It was revealed that the activities of all the dipeptides tested singly or as a mixture was greater than that of glycine for a number of the organisms. The assumption was that these peptides were utilized directly. An interesting observation is the activity of hippuric acid, a peptide of benzoic acid and glycine, (19). The apparent concentration of glycine determined microbiologically in untreated urine was found greater than that in the hydrolyzed urine suggesting that hippuric acid, a normal constituent of urine, was more active microbiologically than glycine.

Some of the work of Simmonds et al. (17), gives essentially the same picture. Some organisms prefer peptides; others prefer amino acids. A strain of Escherichia coli which required exogenous source of proline utilized the peptides of proline more effectively than proline. A second strain utilized proline peptides much less readily. An isolation had been made on a bacillus (18), which showed a pronounced nutritive preference for a peptide, L-leucylglycine rather than a mixture of amino acid of which the peptide is composed.

In studies of Lactobacillus delbrueckii - 3 (14) on requirements the distinctive feature found was the organism's efficient utilization of peptides as a source of essential amino acid in contrast to its inability to utilize free amino acid effectively. Partial hydrolysates were also found as more effective sources of the individual amino acids than were the complete hydrolysates of proteins. Working with strains of Rhizobium melilote, Jordan et al. (6), found that growth of the organism was stimulated when the cultures were grown on media supplemented with glycylglycine as compared with turbidity observed when only glycine was added.

In the investigations of Peters et al. (13, 15), the most active peptide, a histidine peptide acting on L. delbrueckii, contained amino acids which do not occur in proteins, a fact that indicates conclusively that these peptides were not incorporated into proteins of the test organism. Further, Ikawa et al., (4) working also with L. delbrueckii, found that the growth time may be reduced by the addition of crude material such as yeast and liver extracts. Tests on more purified fractions revealed that one category of growth stimulating substances was the peptides present in enzymatic digests of proteins, the other category being the degradation products of ribonucleic acids.

The stimulatory effect of peptides has also been demonstrated in the overcoming of added inhibitors. For example, the growth of L. delbrueckii was shown to be inhibited by glycyl-L-tryptophan and glutathione (14). Partial hydrolysates of proteins effectively prevented these inhibitions, the activity of the partial protein hydrolysates being ascribed to the presence therein of unidentified peptides.

While there are numerous examples of the stimulatory activity of

peptides, it is well to emphasize that these represent exceptions to the more general rule that peptide sources of amino acids are less efficiently utilized than the free amino acids. Some of the material in the following paragraphs serve to illustrate this general rule.

Peptides were shown to serve very inefficiently as far as transfer from cell to cell is concerned (20) A convincing piece of argument was forwarded by Halvorson et al. (21), in support for the hypothesis that free amino acids are the direct precursors of proteins. In Saccharomyces cerevisiae, none of the amino acids were depleted from the cells when the utilization of one of them for growth or enzyme formation was prevented by the presence of a synthetic analogue. Therefore, it was concluded that, "no appreciable quantity of intermediate precursors appeared to be involved in the synthesis of the enzyme or other proteins except for possible precursors already so complex as to require the entrance of the amino acid whose utilization was blocked."

Results from various groups of workers (22-29), give further evidence that where a peptide serves as the source of the growth-limiting amino acid, it does so only after hydrolysis.

Studies made by Simmonds et al. (18, 24, 27) using strains of E. coli revealed that peptides of leucine and peptides of phenylalanine are less active than the corresponding amino acids. Further evidence is found in the work of Hoberman and Stone (28). In studies on the utilization of proline peptides by prolineless mutant, enzymatic cleavage of all peptides tested was prerequisite to the utilization of the amino acid moities in further metabolic reactions. The greater activity of the proline peptides was explained as due to "sparing" of peptidic proline from degradation of bacterial enzymes.

In an investigation (23), considerable variation was found in the utilization of leucine peptides by Lactobacillus arabinosus and Streptococcus faecalis and the difference in the effectiveness of such peptides in replacing an essential amino acid was taken to reflect the differences in the rate of cleavage of the peptides by the bacterial peptidases.

From the literature reviewed, it may be safely concluded that the stimulatory activity of peptides under certain conditions is established. In attempting to answer why they are stimulatory, a number of explanations were suggested: (a) The direct incorporation of peptides into cellular protein synthesis by the organism, (b) the possible destruction of the free amino acid but not of its peptide, and (c) amino acid in peptide linkage must be in a more active form or transferred more readily by the mechanism of transpeptidation.

Many investigations, some of them reviewed above, contradict the direct incorporation theory, but since there is evidence for direct incorporation, it may mean that there exist particular combinations of amino acids bound in peptide linkage which may be utilized in metabolism prior to enzymatic hydrolysis. The second mechanism proposed explains clearly a few instances of peptide stimulation (7, 11), but in most cases, this theory does not find support. In other words, this theory can account for a few but not all cases of peptide stimulation.

In many instances where neither direct peptide incorporation nor free amino acid destruction is the likely mechanism which explains peptide stimulation, transpeptidation may be the answer.

It is now known that peptide bonds may be broken and reconstituted by other processes than by simple hydrolysis and its reversal. For example, an amide, which is not a peptide, may participate in transfer

reactions.

The elongation of peptide chains by transpeptidation has been demonstrated in model substances and is represented by the following type equation.

$$RCONH_2 + N^{15}H_3 \rightleftharpoons RC(OH)(NH_2)(N^{15}H_2)$$
$$\rightleftharpoons RCON^{15}H_2 + NH_3$$

Fruton (30, 31), has considerably extended the significance of this equilibrium by showing that not ammonia alone but amino acids and peptides also may be exchanged *in vitro*. Although the reactions are no more than mere models since the substances involved are not for the most part physiological, these models are, nevertheless, highly suggestive.

Considering the thermodynamics of peptide synthesis, transpeptidation seems a very likely mechanism in peptide utilization. From both the theoretical point of view and from experiments carried out *in vivo* and *in vitro*, energy must be supplied in order to synthesize peptide bonds and proteins. Since the condensation of the two peptide to a larger peptide is energetically easier than the condensation of two amino acids to a dipeptide, the largest "hump" in the biosynthesis of protein is in the formation of small peptides from the free amino acids. Transpeptidation proceeds apparently without considerable variation in free energy since it simply replaces one peptide bond by another. It is thus conceivable that certain molecules already possessing a peptide bond are ready *in vivo* to serve as nuclei for formation of larger molecules.

The problem of how the peptides perform their role in the biological systems is thus still not fully understood. Part of the present studies have been aimed at revealing more examples of the utilization of various

peptides under inhibited and non-inhibited conditions with the ultimate purpose of finding some clues towards the mechanism of their utilization. In addition to the various dipeptides, a new class of synthetic compounds are included in the studies. These are the glyconyl peptides--peptides of aldonic acids coupled through their carboxyl group to the alpha-amino group in a stable -CONH- linkage.

CHAPTER II

GENERAL PROCEDURES

Microorganisms:

The lactic acid bacteria used in the studies were:

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

Leuconostoc citrovorum (ATCC 8021)[2]

Lactobacillus arabinosus 17-5 (ATCC 8014)

Lactobacillus delbrueckii (L. d. III Henneberg)[3]

Streptococcus faecalis R (ATCC 8043)

Lactobacillus casei (ATCC 7469)

These organisms were kept in agar stab cultures (Appendix A) and were transferred to fresh agar medium about every two weeks. Following every transfer, the cultures were incubated at 37°C for approximately 48 hours, and were stored at 4°C.

For experimental work, the organism was transferred into a test tube containing 2.0 ml. of a sterile liquid transfer medium (Appendix A) followed by incubation at 37°C for 18 - 24 hours. The growing cells were prepared for use as inocula by centrifuging them down, removal of the of the supernatant, and resuspension of the cells in 0.9 percent KCl

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[1]Identified as Pediococcus cerevisiae by C. S. McCleskey, J. Bacteriol., 64, 140 (1952).

[2]The name of Streptococcus equinus was suggested by Felton et al., J. Bacteriol., 65, 482 (1953).

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

solution. From a sterile syringe, a drop of the suspended cells was added to each assay tube.

Basal Medium:

The medium (Appendix B) used in all studies was an all-K modification (32) of the uniform medium recommended by Henderson and Snell (33).

In preparing the medium, the growth-limiting amino acid was omitted. Further specific changes in the details will be described later, if necessary, in connection with the experimental work. All studies were conducted at pH 7.0 unless otherwise specified.

Experimental Techniques:

Preparation of experiments

Racks each containing sixty 18 x 150 mm rimless tubes with six tubes per row were employed. Various procedural plans were employed in the different experiments. In general, the growth-limiting amino acid or peptide was dispensed in graded amounts to the tubes in a row and water was used in bringing the volume to a final volume of 2.0 ml. The additions of the solutions were carried out by means of a Cannon automatic dispenser.

Sterilization

Following dispensing of the solutions, the tubes were sterilized in a preheated autoclave for 5 minutes at 15 lb. pressure (121°C). The pressure was then rapidly reduced to atmospheric pressure and the racks removed immediately from the autoclave and cooled to room temperature.

Inoculation and incubation

The bacterial cells which had been grown for about 18 hours preceding the experiment preparation were collected by centrifugation and were

suspended in 0.9 percent KCl solution. This suspension of bacterial cells in KCl was used as the inoculum. One drop of the suspension was added to each tube by means of a sterile syringe. The tubes were then incubated at 37°C for 64-72 hours.

Measurement of growth

Using acid production by the bacteria as an index of growth, the measurement of growth was accomplished by titration of the acid with approximately 0.05 N KOH. The electrometric cell consisted of a 1.0 N calomel electrode as a reference and a quinhydrone electrode as an indicator. The amount of base used for titration to pH 7.3 was expressed in terms of titration counts, 100 counts of which correspond to about 4.0 ml of the base. The titration was carried out by means of a Cannon automatic titrator.

CHAPTER III

MICROBIOLOGICAL ACTIVITY OF GLYCONYL PEPTIDES

Introduction

The glyconyl peptides, a class of carbohydrate-amino acid compounds, synthesized by Doherty (34), are aldonic acids coupled through their carboxyl group to the alpha-amino group of the amino acids in a stable -CO-NH- linkage. This class of compounds is of interest in connection with the known destructive effect of autoclaving amino acids and proteins in the presence of different carbohydrates. In controlled experiments (35) in which protein hydrolysates of known biological value were dried in the presence of glucose, browning and loss of nutritive value occurred even under mild conditions. It is of interest that microbiological assays yielded reduced amino acid values, but the reduction was not as great as was indicated with animals. It is possible that the amino acid-sugar reactions form compounds essentially similar to the glyconyl peptides which may be less available to the microorganisms than the free amino acids themselves.

Specific procedure and results

To check for the possibility that the glyconyl peptide-like compounds are a class of stable product in the heating of amino acids and proteins with sugar, the simple procedure of subjecting the peptides to the usual conditions of preparing protein hydrolysates- 3N HCl and

hydrolysis time of 15 hours - was employed. Assays for purity were set up. The results are summarized in Table 1.

Table 1

	Percent
D-Arabonyl-L-leucine ethyl ester	100
D-Gluconyl-L-phenylalanine ethyl ester	100
D-Arabonylglycine ethyl ester	84
D-Gluconylglycine ethyl ester	91
D-Arabonyl-L-tyrosine ethyl ester	88
D-Galactonyl-L-tyrosine ethyl ester	92
D-Arabonyl-L-glutamic diethyl ester	87
D-Gluconyl-L-glutamic diethyl ester	92

To test for biological activity, the ester group of the glyconyl peptide ester had to be removed since an ester form of an amino acid peptide, in contrast to unsubstituted forms of peptides, was found to be much less active for biological growth (29).

To remove the ester group, the glyconyl peptide ethyl ester was dissolved in 20 ml. of water and 9 ml. of 1N KOH were added. After ½ an hour at room temperature, the solution was neutralized with 1N HCl to pH 7.0.

The glyconyl peptide solution at the concentration equivalent to the standard growth-limiting amino acid, was added to each row, so that six tubes in the row received respectively 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 ml. Distilled water was added to bring the volume to 1.0 ml. per tube.

Standard rows containing graded amounts of growth-limiting free amino acid were set up in the same way. The basal medium with the growth-limiting amino acid omitted was then added to each tube at 1.0 ml. per tube to bring the final volume to 2.0 ml.

The concentration of the standard amino acids used are summarized in Table 2.

Table 2

Amino Acid	Conc. of Standard ug./ml. of the L-form
L-tyrosine	10
L-leucine	15
glycine	10
DL-phenylalanine	10
DL-glutamic	50

Each of the glyconyl peptides were tested for biological activity with a number of organisms. The results as shown in Figs. 1-4 revealed that the peptides differ in activity from organism to organism and from peptide to peptide. The magnitude of biological activity as compared with the standard free amino acid ranged from negligible to nearly equivalent activity depending on the peptide and the organism involved. In all cases, the glyconyl peptides were shown to be less available to the microorganisms than the free amino acids under conditions that are known to be optimal for the utilization of the growth-limiting amino acids.

A study was made on the effect of autoclaving on the availability of the growth-limiting amino acid leucine and its glyconyl peptide source. The same general procedure as that employed in the testing for the biological activity was used except that in this investigation, the growth-limiting amino acid sources were not added to half of the tubes until all other constituents of the medium had been autoclaved. After the tubes were cooled, the limiting amino acid sources were added aseptically, i.e., by filtering the solution through the Seitz filter and pipeting into the tubes the graded amounts of the limiting nutrient by means of sterilized pipets.

Fig. 5 shows that when the limiting leucine or the glyconyl-leucine is added aseptically, L. arabinosus gave greater growth response, especially in the region of higher concentrations.

Discussion

In the assay of glyconyl-peptide hydrolysates, a less than 100 percent purity can be interpreted in two ways: (a) that the -CONH-bond in glyconyl peptide is more resistant to ordinary conditions of hydrolyzing proteins or (b) that the glyconyl peptide preparations are not chemically pure. If all the glyconyl peptides were found to be significantly less than 100 percent pure, it would be well to suppose that the -CONH- bond of the glyconyl peptide is more resistant to hydrolysis than the -CONH- bond joining two amino acids. Since there are two of the glyconyl peptides that gave essentially 100 percent purity in the assays, it is logical to assume that the lower values obtained with the other six glyconyl peptides are due to impurities in the peptide samples. Although the -CONH- bond of the glyconyl peptide was shown to be no more resistant to acid hydrolysis than ordinary peptide bonds, this finding

still does not rule out the possibility of the formation of sugar-amino acid compounds similar to the glyconyl peptide under ordinary sterilization conditions. Assuming that such reactions may actually take place, it would be well to know if compounds such as the glyconyl peptides possess biological activity. Obviously, where the glyconyl peptides possess less or no biological activity, an assay with the growth-limiting amino acid tied up as glyconyl peptide-like compounds would give erroneously low results.

The test for activity shows a great variation of the response of various organisms to various peptides. For example, L. arabinosus utilizes growth-limiting quantities of D-arabonyl-L-leucine almost as well as free leucine, but Leuc. mesenteroides gives negligible response to the same glyconyl peptide. This difference in response may be due to the ability of L. arabinosus to produce an enzyme which catalyzes the utilization of D-arabonyl-L-leucine, but which ability Leuc. mesenteroides does not possess. A similar reasoning can easily be applied to all the other glyconyl peptides studied.

The glyconyl peptides of the same amino acid but different sugar residues were found to differ in their activity. It was revealed that in all cases, the peptide with the hexose sugar residue gave greater growth response than the peptide with the pentose residue with the same organism. Why this is so is not clearly understood, since the glucose in the medium would actually eliminate any concentration effect provided by the small amount of sugar residue introduced with the growth-limiting amino acid source.

In a study of the influence of the amino acid- dextrose reaction on the growth of lactic acid bacteria, Rose and Peterson (36) found

little direct evidence of toxic effects attributable to Maillard products. In fact, their results showed that a medium which had been autoclaved after the addition of dextrose promoted more rapid growth than a medium for which the dextrose had been autoclaved separately.

In the present study of the effects of autoclaving on the media the results revealed that there is some kind of interaction of the growth-limiting free amino acid or peptide source with certain components of the medium when the limiting nutrients are autoclaved with the other components of the medium. These interactions are responsible for the lower growth response of the organism. Although such experiments do not reveal the type and nature of the interactions, it is possible that the free amino acid or the peptide source reacted with glucose to form some kind of Maillard product which may inhibit growth because of the loss of the available limiting nutrient.

Summary

The glyconyl peptides, a class of synthetic sugar-amino acid compounds, were tested for biological activity. The results revealed that the activity as compared with the free amino acid ranges from negligible to nearly-equivalent depending on the organism and the peptide involved.

A purity check shows that the -CONH- bond of the glyconyl peptide is not more resistant to acid hydrolysis than ordinary peptide bond.

Study on the effect of autoclaving by adding the limiting nutrient aseptically after the other components were sterilized indicates that Maillard products might lower assay values by rendering the limiting nutrient unavailable or less available to the organism.

CHAPTER IV

STUDIES ON THE UTILIZATION OF FREE AMINO ACIDS, AND THEIR CORRESPONDING DIPEPTIDES, AND GLYCONYL PEPTIDES

Introduction

In the studies of the utilization of amino acids by lactic acid bacteria, many interrelationships of various types have been found. From such studies, conditions involving inhibitions brought about by the imbalance of the amino acids in the medium have been recognized and these have been extended to the study of peptide activity.

In the present investigations, interrelationships involving the "limiting" amino acids glycine and tyrosine with a number of "modifying" amino acids were studied. The term "limiting" is used to designate the amino acid present in growth-limiting amounts and the term "modifying" refers to the amino acid for which the effect on the response of the growth-limiting one is being studied. The purpose of such preliminary interrelationship studies was to identify especially-striking conditions of inhibitions brought about by merely altering the concentration of certain modifying amino acids in the medium, and then to employ these conditions in the studies of the utilization of the peptide sources in the forms of glyconyl peptides and dipeptides.

Specific Procedure and Results

The general procedure employed in the survey of amino acid inter-

relationships was the measurement of the growth response of an organism to the growth-limiting amino acid at different levels of concentration of the modifying amino acid. In the general survey, the normal Henderson and Snell concentration and 1/10 of the Henderson and Snell concentration were used. The normal Henderson and Snell concentration of an amino acid referred to in these studies is the concentration of the amino acid as recommended for use in a uniform medium by Henderson and Snell (33), (Appendix B). In further discussion, the abbreviation H-S will be used. Concentrations other than the prescribed amounts of amino acids will be identified as fractions or multiples of the H-S concentrations. Where the results indicated a higher degree of interrelationship, further studies were conducted over a wider range of concentrations of the modifying amino acids.

The interdependence of tyrosine as the limiting amino acid with four different "modifying" amino acids was investigated using L. delbrueckii-3 and Leuc. mesenteroides.

The following solutions were prepared to render possible the use of a uniform medium in the interrelationship studies:

1) Complete amino acid mixture minus tyrosine, glycine, threonine, phenylalanine, and proline.
2) Solution of glycine, proline, phenylalanine, and threonine all at the normal H-S concentration.
3) Solution of glycine, proline, phenylalanine at the normal H-S concentration and threonine at 1/10 of the normal H-S concentration.
4) Solution of glycine, proline, and threonine at the normal H-S concentration and phenylalanine at 1/10 of the normal concentration.

5) Solution of glycine, threonine, and phenylalanine at the normal H-S concentration and proline at 1/10 of the normal H-S concentration.

6) Solution of proline, threonine, phenylalanine at the normal H-S concentration and glycine at 1/10 of the normal H-S concentration.

As in routine procedure, the limiting amino acids were added in graded amounts to each row and the volume of all tubes brought up to 1.0 ml. with H_2O. The uniform medium containing amino acid mixture, no. 1 was added at 0.6 ml. per tube. The modifying amino acid mixes No. 2 - No. 6 were then added at 0.4 ml. per tube making the total volume 2.0 ml.

The results are shown in Table 3.

Table 3

Types of Amino Acid Interrelationships	Organism	Limiting Amino Acid	Modifying Amino Acid
1. Inhibition of the utilization of a limiting amino acid by a high concentration of a modifying amino acid	L. delbrueckii-3	L-tyrosine	DL-phenylalanine
	Leuc. mesenteroides	glycine	DL-alanine L-cystine L-glutamic
	Leuc. citrovorum	glycine	L-cystine L-leucine
2. Inhibition of the utilization of a limiting amino acid by a low concentration of a modifying amino acid	Leuc. mesenteroides	L-tyrosine	DL-phenylalanine L-proline DL-threonine glycine
	L. delbrueckii-3	L-tyrosine	glycine L-proline DL-threonine
	Leuc. citrovorum	glycine	L-glutamic L-arginine DL-isoleucine
	Leuc. mesenteroides	glycine	L-arginine

The interdependence of glycine as the growth-limiting amino acid with several modifying amino acids were investigated using Leuc. mesenteroides and Leuc. citrovorum. The latter organism requires folinic acid which was added at 0.1 μg/tube.

In the studies with Leuc. citrovorum, a number of modifying amino acids were varied individually in the medium. The procedure consisted of obtaining a standard curve using a medium containing the 17 amino acids at the normal H-S concentrations. To study the effect of changing the concentration of one of the 17 amino acids in the medium, duplicate rows containing the modifying amino acid at a different concentration than that used for establishing the standard curve were used. In this study, 1/10 of the H-S concentration was chosen. Table 3 reveals the effect of low concentration of the modifying amino acids on the response to the growth-limiting by Leuc. citrovorum.

The other organism used in the interrelationship study was Leuc. mesenteroides. The effect of a number of modifying amino acids are revealed in Table 3.

The results of the interrelationship studies revealed that there are varying degrees of effect from altering the concentration of an amino acid in the medium. It is also obvious that the kind and magnitude of effect depends upon the organism used, the growth-limiting amino acid and the modifying amino acid involved.

Further studies were conducted using a still lower concentration of phenylalanine in addition to the concentrations employed in the previous studies. Figs. 6, 7, and 8 demonstrate the different effects on three organisms from variation of a modifying amino acid.

Results clearly indicated that a high concentration of phenylalanine

is necessary for the efficient utilization of limiting tyrosine by Leuc. mesenteroides. On the other hand, high concentration of phenylalanine inhibits the utilization of growth-limiting amounts of tyrosine by L. delbruckii-3. With L. arabinosus, however, varying the concentration of the modifying phenylalanine has no effect on the growth of the organism.

Further studies of the glycine-arginine interrelationship was conducted. Table 3 shows that a normal H-S concentration of arginine is favorable to the growth of Leuc. mesenteroides when glycine is limiting, verifying the results obtained earlier in this laboratory by Wold (2). In the present investigation, the results showing response of Leus. mesenteroides to various levels of arginine in the medium indicated that the magnitude of response are of the order:

$$\text{H-S concentration} > 4 \times \text{H-S} > 1/10\ \text{H-S} > 1/20\ \text{H-S}$$

Thus at too high a concentration, arginine becomes toxic to the organism even though a fairly high amount, i.e., the normal H-S concentration, is necessary for its optimal response. This behavior is very similar to that observed by Sirny, et al. (1) for the effect of arginine on proline utilization, and may merely indicate a general toxicity of arginine in high quantities.

After conducting the preliminary interrelationship studies, the next logical step was to employ the conditions of inhibitions brought about by variation of the concentration of a modifying amino acid in the study of peptide utilization. A comparison was made of peptide utilization with the corresponding free amino acid utilization under both inhibited and non-inhibited conditions.

The peptide solutions contain equivalent amounts of the free amino acids. As in other experiments, graded amounts are added to the six

tubes in a row. Duplicate rows of each source of the growth-limiting amino acid were set up, one row containing a high concentration of the modifying amino acid and the other row containing low concentration of the same modifying amino acid. There was, therefore, an alternation of inhibited and non-inhibited conditions on the same rack. To check the reliability of an experiment, duplicate racks were set up in each experiment conducted.

With tyrosine as the growth-limiting amino acid, the following peptides were used as the sources of tyrosine in the study:

L-leucyl L-tyrosine, glycyl L-tyrosine, D-arabonyl-L-tyrosine, and D-galactonyl-L-tyrosine.

Using Leuc. mesenteroides and varying the concentration of the modifying amino acid phenylalanine from 1/20 of the H-S concentration to the normal H-S concentration, results were obtained which revealed some cases of peptide stimulation. Figs. 9a and 9b.

Under uninhibited condition, i.e., at the normal H-S concentration of modifying phenylalanine, all the peptides were less available than the free amino acid tyrosine to Leuc. mesenteroides whether these peptides were the glyconyl peptides or the dipeptides in which the components were only amino acids.

At 1/20 of the H-S concentration of phenylalanine, however, leucyltyrosine produced a stimulatory effect over the free tyrosine at the higher levels of the standard curves. As the graph clearly indicates, leucyltyrosine was rendered less available under inhibited condition, but the effect was not as great as the magnitude of inhibition found with the free tyrosine.

With glycyltyrosine as the growth-limiting source of tyrosine, the

graph shows a high degree of lag even at the normal H-S concentration of phenylalanine.

With the glyconyl peptides, the imbalance of amino acids brought about by lowering phenylalanine also inhibits their utilization, although the magnitude of inhibition is not as great as that demonstrated with the free tyrosine.

The results of this investigation using Leuc. mesenteroides and the various sources of the growth-limiting tyrosine seems to indicate that the free amino acid tyrosine is more sensitive to changes such as alteration of the concentration of another amino acid.

In the studies with L. delbrueckii-3, varying the concentration of phenylalanine from 1/20 of the H-S concentration to the normal H-S concentration, the results also revealed the stimulatory effect of the dipeptides under inhibited conditions. With this organism, the normal H-S concentration of phenylalanine inhibited the utilization of growth-limiting source of tyrosine. From Figs. 10a and 10b, it is evident that the utilization of the dipeptides was negligibly affected by varying the concentration of phenylalanine over a twenty fold range. Unlike the response of Leuc. mesenteroides in which L-leucyl-L-tyrosine was much more effective in promoting growth than glycyl-L-tyrosine, the responses of L. delbrueckii-3 to both peptides are equivalent and both were shown to be stimulatory in contrast to free tyrosine under inhibited condition.

A turbidimetric study was conducted using Leuc. mesenteroides and L-tyrosine in the free and peptide forms. In this method, growth was measured as a function of the turbidity of the solution. This procedure permitted determination of not only the maximum growth, but also the

progress of the growth at certain periods of time, e.g., after fifteen hours of incubation. The results were similar to those obtained from the titrimetric method. In addition, it was revealed that the peptide L-leucyl-L-tyrosine initiates growth after a shorter period than tyrosine. This was in contrast to all the other peptides tested which were shown to be slower in initiating bacterial growth.

A study was made of the response of Leuc. citrovorum to growth-limiting glycine and its peptide sources at two different concentrations of leucine in the medium. The peptide sources of glycine made available for the study were: L-leucylglycine, glycyl-L-leucine, D-arabonylglycine, and D-gluconylglycine. A correction was made for the leucine introduced with glycyl-L-leucine and L-leucylglycine so that the concentration of leucine in the medium for all the growth-limiting amino acid source would be equal. Equivalent amounts of leucine introduced with the peptides glycyl-L-leucine and L-leucylglycine were added to glycine, D-arabonylglycine, and D-gluconylglycine. Using Leuc. citrovorum and varying the concentration of the modifying amino acid L-leucine from 1/10 of the H-S concentration to the normal H-S concentration, results were obtained (Figs. 11a and 11b) which again revealed examples of peptide stimulation.

The results revealed that at both concentrations of L-leucine as the modifying amino acid, the growth-response curves to the peptides glycyl-L-leucine and L-leucylglycine do not possess the lag at low concentration of the growth-limiting amino acid. With the glyconyl peptides, on the other hand, the initial lag is more pronounced than with the free amino acid glycine, as is clearly revealed in the graphs.

Varying the concentration of the modifying amino acid arginine

from 1/20 of the H-S concentration to the normal H-S concentration, a study was made of the responses of Leuc. mesenteroides to growth-limiting quantities of glycine and its dipeptide sources at pH 7.0 and pH7.5. The results are summarized in Figs. 12 and 13. The pH effect is not very pronounced, although it is seen that pH 7.0 seems to be more optimal for the utilization of free glycine and its glyconyl peptides. In this investigation, the glyconyl peptides were found to exhibit greater biological activity than was expected; however, this was suspected to be due to the slow hydrolysis of the peptide when stored in 1N KOH. This can very likely account for the comparable magnitude of inhibition as that of the free amino acid glycine under suboptimal condition.

In the preliminary interrelationship studies, a 1/10 of the normal H-S concentration of L-cystine was found to promote greater growth response with both Leuc. mesenteroides and Leuc. citrovorum. An investigation was conducted in which the responses of these organisms to free glycine and its peptides at different concentrations of cystine were noted. The results are shown in Figs. 14 and 15. The low concentration used in this investigation was 1/20 of the normal H-S concentration. With Leuc. mesenteroides, the picture is essentially the same as that revealed with the study in which L-arginine was the modifying amino acid except that here, the interrelationship is not as striking. A twenty-fold difference in concentration of cystine caused only small difference in response. Secondly, there was practically no difference in the degree of inhibitions found with the dipeptides, the glyconyl peptides, and the free amino acid glycine. There is no other more feasible explanation for the great activity of the glyconyl peptides

than that complete hydrolysis must have taken place after storage of the peptides for some time (several weeks) in 1N KOH.

With Leuc. citrovorum, the lags in the growth-response curves for free glycine and the glyconyl peptides are possibly due to the destruction of the folinic acid, which is required by this organism, either from accidental autoclaving at about 22 lbs. pressure at a considerably higher temperature or from keeping folinic acid in solution for too long a period of time. It is, however, of interest to note that in spite of the existent inhibition in the experiment, the dipeptides gave good growth curves at both concentrations of L-cystine: at 1/20 of the normal H-S concentration and at the normal H-S concentration.

Discussion

A number of important factors must be considered in the study of the free amino acids, their dipeptides, and their glyconyl peptides. Conditions such as pH, autoclaving time and temperature, incubation period, concentration of inoculum, etc. all affect measurably the response of an organism to a limiting nutrient. In the present investigations where the main object was to study the effect of varying the concentration of certain modifying amino acids, these other conditions were kept as constant as possible.

The studies revealed many examples of peptide stimulation as demonstrated by dipeptides. In the case of the utilization of L-leucyl-L-tyrosine by Leuc. mesenteroides, the stimulatory effect may not be considered a true one, for as clearly shown in Figs. 9a and 9b, L-leucyl-L-tyrosine gave greater growth response than free tyrosine only when the requirement for the high amount of DL-phenylalanine is not met. Also evident from the same figure is that the peptide, like the free

amino acid, also gave greater growth response at high concentration of DL-phenylalanine than at low. Therefore, the apparent stimulatory effect of L-leucyl-L-tyrosine over free tyrosine at low DL-phenylalanine in the medium appears to be due to the lesser effect of a low DL-phenylalanine concentration on L-leucyl-L-tyrosine utilization than on L-tyrosine utilization.

On the other hand, glycyl-L-tyrosine gave a very high lag in the dose-response curve and is even less utilized than the glyconyl peptides at low concentrations. This suggests that at low concentration of glycyl-L-tyrosine, something in the medium strongly inhibits its utilization and such inhibition is slowly overcome as the concentration of glycyl-L-tyrosine increases.

How a peptide is utilized and whether or not it possesses an apparent or a true stimulatory effect also depend to a great extent on the organism. With L. delbrueckii-3 (Figs. 10a and 10b), a lower concentration of DL-phenylalanine promoted greater response to growth-limiting amounts of L-tyrosine. When the dipeptides were studied using media containing the two different concentrations of DL-phenylalanine, the growth responses differ negligibly for each of the dipeptides L-leucyl-L-tyrosine and glycyl-L-tyrosine. The results revealed that these dipeptides are both utilized to about the same degree and are both insensitive to a change of concentration of DL-phenylalanine even as much as twenty-fold. A distinct interrelationship, however, is found with the free amino acid. At the normal H-S concentration of phenylalanine, the growth is much reduced with free tyrosine. Under such inhibited conditions brought about by altering an amino acid of the medium, the peptides L-leucyl-L-tyrosine and glycyl-L-tyrosine were found to be

more utilized than the free L-tyrosine and thus may be said to possess apparent stimulatory effect.

The structural similarity of phenylalanine and tyrosine might lead one to suspect that DL-phenylalanine may be utilized by Leuc. mesenteroides for the synthesis of L-tyrosine. That such is not the case is supported by the study of L-tyrosine utilization at varying concentrations of DL-phenylalanine. If DL-phenylalanine had acted as the direct precursor in the synthesis of L-tyrosine, the blanks would have been higher where the medium contains a ten-fold and a twenty-fold higher concentration of phenylalanine. Therefore, the fairly high concentration of DL-phenylalanine required for optimal utilization of limiting L-tyrosine serves some function other than the synthesis of tyrosine.

The tyrosine - phenylalanine interrelationship with L. delbrueckii-3 can be more easily explained. Since a high concentration of DL-phenylalanine inhibits tyrosine utilization, this may be explained as one of the many examples of competitive type of inhibition due to structural similarity. The dipeptides containing L-tyrosine were apparently not inhibited by DL-phenylalanine.

Perhaps more closely approaching true stimulatory effects are the responses of the organisms Leuc. mesenteroides and Leuc. citrovorum to dipeptides of glycine where these dipeptides take the place of glycine in serving as the source of the limiting amino acid. Figs. 11, 12, 13, 14, and 15 clearly show that where these dipeptides were used in place of glycine, greater growth responses were obtained under all concentrations of the modifying nutrient. The lag in the growth curve at lower concentration of glycine so characteristic of the glycine standard curve was eliminated by supplying glycine in the form of di-

peptides. It is a logical conclusion that the elimination of lag in the growth curves is due to the overcoming of inhibitions existent at low concentration of the growth-limiting mutrient when the latter is supplied in the free form.

The suggestion that the dipeptides are relatively insensitive to changes in conditions is further supported by the kind of results obtained in Fig. 15. Leuc. citrovorum responded to the dipeptides very well but had big lags in the curves where the growth-limiting nutrients were in the form of the free amino acid or its glyconyl peptides.

If the specific change in conditions in this case was the destruction of folinic acid in the medium, which has been indicated as a possibility, this would suggest that the requirement for Leuc. citrovorum for folinic acid is less when the amino acid is supplied in the form of dipeptides. Stating this in another way, the function of folinic acid in Leuc. citrovorum would appear to be more intimately involved in the utilization of the free amino acids than of the dipeptides.

Summary

Studies of the effects of a number of different amino acids on the utilization of glycine and L-tyrosine were conducted. Among the more striking interrelated effects were those of tyrosine and phenylalanine and of glycine and arginine, and these were studied more extensively.

Different effects were manifested with different organisms. When L-tyrosine is the growth-limiting amino acid, a higher concentration of DL-phenylalanine was found to inhibit growth with L. delbrueckii-3 but was shown to promote better growth with Leuc. mesenteroides.

Peptide stimulatory effects were demonstrated in many instances. L-leucyl-L-tyrosine possesses apparent stimulatory effect at suboptimal

condition for both L. delbrueckii-3 and Leuc. mesenteroides. Glycyl-L-tyrosine, however, possesses apparent stimulatory effect with only the former and not with the latter organism.

Dipeptides of glycine were found to give better growth responses than glycine under all conditions and with all organisms studied.

The glyconyl peptides were found to be affected by inhibited conditions in every case though the magnitude of inhibitions was to a proportionally lesser degree than with the corresponding free amino acids themselves.

CHAPTER V

GENERAL DISCUSSION

The studies of amino acid interrelationships and peptide utilization reported here have yielded further evidence for a high degree of interrelationships between amino acids and for a varied degree of availability of peptide-bound amino acids. The results are essentially consistent with the various observations on peptide utilization reported in the literature, for here evidence has also been found for both decreased availability as well as apparent stimulatory effect of the amino acids in the peptides.

Where the peptides are less available, as in the utilization of L-leucyl-L-tyrosine and glycyl-L-tyrosine by Leuc. mesenteroides at relatively high concentration of DL-phenylalanine and by L. delbrueckii-3 at a low concentration of the same amino acid, the explanation of the reduced availability may be simply explained on the basis of limited ability on the part of the organisms to hydrolyze the peptides.

Under inhibited conditions brought about by altering the concentration of a modifying amino acid in the medium, stimulatory effects of some peptides were demonstrated. At a relatively high concentration of DL-phenylalanine, L-leucyl-L-tyrosine and glycyl-L-tyrosine were found to be more efficiently utilized than the free amino acid L-tyrosine by L. delbrueckii-3. On the other hand, a high concentration of DL-phenylalanine is required for the efficient utilization of free

tyrosine by Leuc. mesenteroides. When the concentration of DL-phenylalanine was reduced from the normal H-S concentration to 1/20 of the H-S concentration, the peptide source, L-leucyl-L-tyrosine, was found to be stimulatory. The inhibitory effects caused by some amino acids at certain concentrations apparently do not affect the utilization of the peptides as much as they affect the free amino acids.

Although peptide stimulatory effects may seem to indicate a direct incorporation mechanism, this is not in itself the ultimate proof that such mechanism exists. Specific instances where a feasible explanation was given (7, 28) argue against direct utilization of peptides, at least for some of the cases of peptide stimulation.

It should be pointed out that it is highly probable that the conditions existing as a consequence of the balance of constituents at the H-S concentrations in the media are not truly non-inhibited conditions. In fact, it is recognized that there exist in the medium other already-identified inhibitions and that many more unidentified ones may also exist. Thus, even under the conditions which have been treated as "optimal" for the utilization of the free amino acids, there may be imbalances which interfere with their utilization. This would account for the higher activity of the glycine peptides under all conditions tested, although this may not be the only explanation.

That various dipeptides are utilizable when the free amino acid nutrient is absent from the medium may seem to indicate that hydrolysis prior to incorporation occurs. Since the configuration in the vicinity of a peptide bond influences the free energy of formation of that bond, the difference in the degree of utilization of various dipeptides should not be unexpected if a hydolytic mechanism is opera-

tive. Peptide bonds, however, may be broken and reconstituted by other processes than by simple hydrolysis and its reversal, e.g., by transfer reactions which are energetically easier. If a transfer reaction is operative, the glyconyl peptides should have a comparable efficiency in supplying the limiting nutrient as the dipeptides provided that there is no interference by the carbohydrate component of the molecule. That the activity of the glyconyl peptides was low would support the view that peptide activity may not be attributable entirely to the -CONH- linkage or that these dipeptides may be functioning in protein synthesis not only as amino acid donors, but also serve as amino acid acceptors in the cell and thus be built up to proteins. The latter proposition connotes a direct uptake of the peptides by the cell followed by synthesis of proteins in the cell. As pointed out by Dunn et al. (37), although there is a prevalent concept that competitive inhibition of the utilization of exogenous nutrients by structural analogues frequently results from specific inhibition of the uptake of nutrients by the cell, specific inhibition of the metabolic reactions in the cell may be the primary phenomenon involved.

The results of the present study also demonstrate the insensitiveness of peptides to changes in conditions such as pH, and concentrations of other amino acids in the medium and possibly the sparing effect for a necessary nutrient. Whatever the mechanism of their utilization is, peptides must be used via a different route than the component free amino acids. That different dipeptides differ in their availability and sensitivity to changes in the medium may mean that there are different routes for different peptides. That a peptide source of an amino acid can satisfy the requirement for that amino acid by an organism

may indicate that in the process of protein biosynthesis, both peptides and amino acids form common "active intermediates" which are the more direct precursors in the pathway of protein biosynthesis. That the peptides are stimulatory in some instances may be due simply to the more specific conditions which must be met in order for the free amino acid to form the "active intermediate." In certain unfavorable conditions, the amino acid may be destroyed or rendered unavailable by some other component in the medium, which might be another amino acid.

Biosynthesis of protein involves the question of whether small or large peptides are needed in the biosynthesis or if some template mechanism is operative. The results presented in this thesis support the view that peptides do play certain roles in the biosynthesis of protein. As pointed out by Christensen (38), the very low level of tissue peptides, aside from a few specialized structures, had led to uncertainty as to the place of peptides as intermediates in the synthesis of proteins. It is not unlikely that the intermediates which are the direct precusors of proteins exist but they may be short-lived or transient as are many intermediates.

The results provided many examples of differences in response of organisms to the same nutrient. L. arabinosus utilizes D-arabonyl-L-leucine many times more effectively than Leuc. mesenteroides. In the response of L. delbrueckii-3 and Leuc. mesenteroides to growth-limiting tyrosine dipeptides L-leucyl-L-tyrosine and glycyl-L-tyrosine at different phenylalanine level in the medium, both peptides were utilized to about the same degree by L. delbrueckii-3 but not by Leuc. mesenteroides. Presumably this should be explainable in terms of the ability of an organism to produce specific enzymes to catalyze the utilization

of a nutrient, especially when it is not in the free form. Considering the specific sequences of amino acid units in the peptides isolated from various proteins, there might conceivably be a certain stage in the biosynthesis of proteins where a certain amount of selection takes place of the amino acids and peptides which condense to form proteins. As a hypothesis, Lipmann (39) endows the enzyme involved with a selectivity, and assumes that amino-acid-specific activation spots are lined up on a structure in the demanded sequence. From his point of view, the responsibility for the specific amino acid sequences in proteins lies in the specific structure of the enzyme at the sites of protein synthesis.

The concept of peptide stimulatory effect is not new. As early as 1944, the term "strepogenin" was proposed by Sprince and Woolley (40) to characterize the growth stimulatory effect of hydrolysates of various proteins. Woolley's later investigations (41, 43) revealed strepogenin activity occurring in synthetic dipeptides and tripeptides. Since the studies of Woolley (40 - 44) up to the recent work of McAnelly et al. (45), it has been the concept of many that the source of strepogenin activity or peptide stimulatory effect is a property of some specific peptides. In fact, the presumption that strepogenin is a single moiety had led to many unsuccessful efforts to identify it chemically.

That peptide stimulatory effect is a phenomenon which can not be attributed solely to the specific peptide found to be growth-promoting is supported by the results obtained in this laboratory. The results indicated that peptide stimulatory effect is a rather general property which, as suggested by experimental evidence, is dependent upon the insensitivity of peptides to changes in the bacterial medium.

Though the concept of peptide stimulatory effect is established, the problem of the mechanism of uptake and utilization of peptides remains in question. The various proposed mechanisms all seem to have some degree of support. Although there is no direct evidence that a direct uptake mechanism does exist, such a mechanism can not be considered unlikely until the inability of a cell to assimilate exogenous peptides will be proven without doubt. The second proposed mechanism is the protection of peptides from various inhibiton conditions.

This finds support in the results of the studies reported in this thesis. Many examples of apparent stimulatory effects of peptides have been described and all are consistent with the explanation based on their invulnerability to inhibitions existent in the medium. The present work does not exclude the possibility that a third proposed mechanism, transpeptidation, may be involved. If transpeptidation is indeed the primary mechanism in peptide utilization, it is quite likely that this mechanism would not be affected by inhibitions operating at the free amino acid level.

CHAPTER VI

GENERAL SUMMARY

A number of glyconyl peptides tested for biological activity were found available to the microorganisms in varying degrees, ranging from negligible or no activity to nearly equivalent activity as compared with the corresponding free amino acids. A purity check was made on each of the eight glyconyl peptides. In the study conducted on the effect of autoclaving a limiting nutrient with the other components of the bacterial medium, the results indicated that interactions had possibly rendered the growth-limiting nutrient less available to the organism involved.

Studies made on the effect of a number of modifying amino acids on the responses of the microorganisms to limiting amounts of the amino acids glycine and L-tyrosine revealed many different types and varying degrees of interrelationships. In two instances, that of arginine-glycine and tyrosine-phenylalanine interrelationships, more extensive studies were conducted.

Conditions of inhibition brought about by changing the concentration of the modifying amino acids were found in the interrelationship studies. These inhibited conditions and the non-inhibited conditions were employed in further studies of the utilization of free and peptide-bound L-tyrosine and glycine. Peptide stimulatory effects were demonstrated with the dipeptides in many instances. Under every condition

employed, the glyconyl peptides were utilized to a lesser extent than were the constituent free amino acids.

Some of the possible mechanisms by which these varying degrees of utilization of the free amino acids and their related peptides may be explained and have been discussed. It has been suggested that the stimulatory effects observed for peptides are attributed to the insensitivity of peptides to various inhibitions operating at the free amino acid level.

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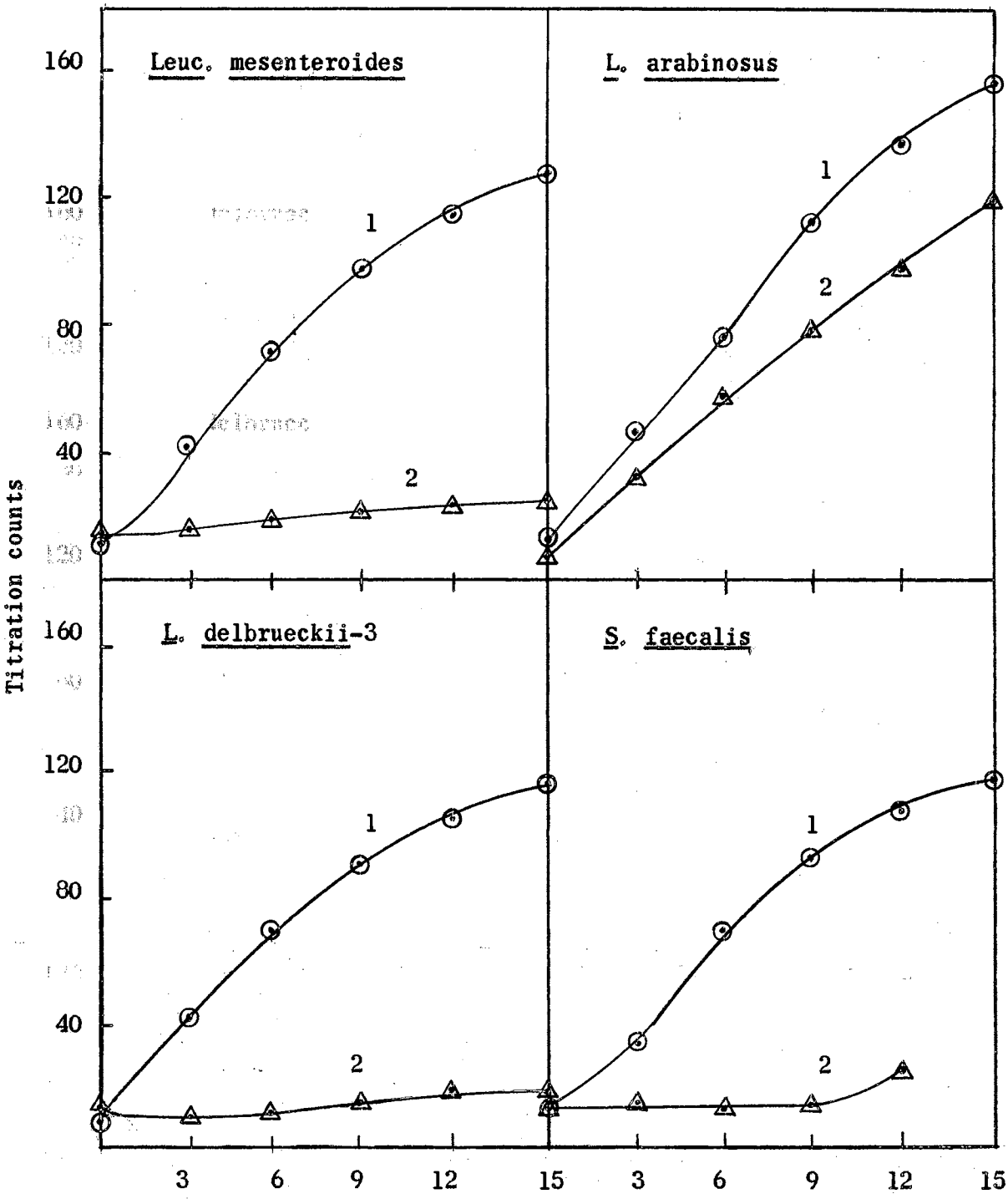
Figure 1

Utilization of L-Leucine and D-Arabonyl-L-Leucine

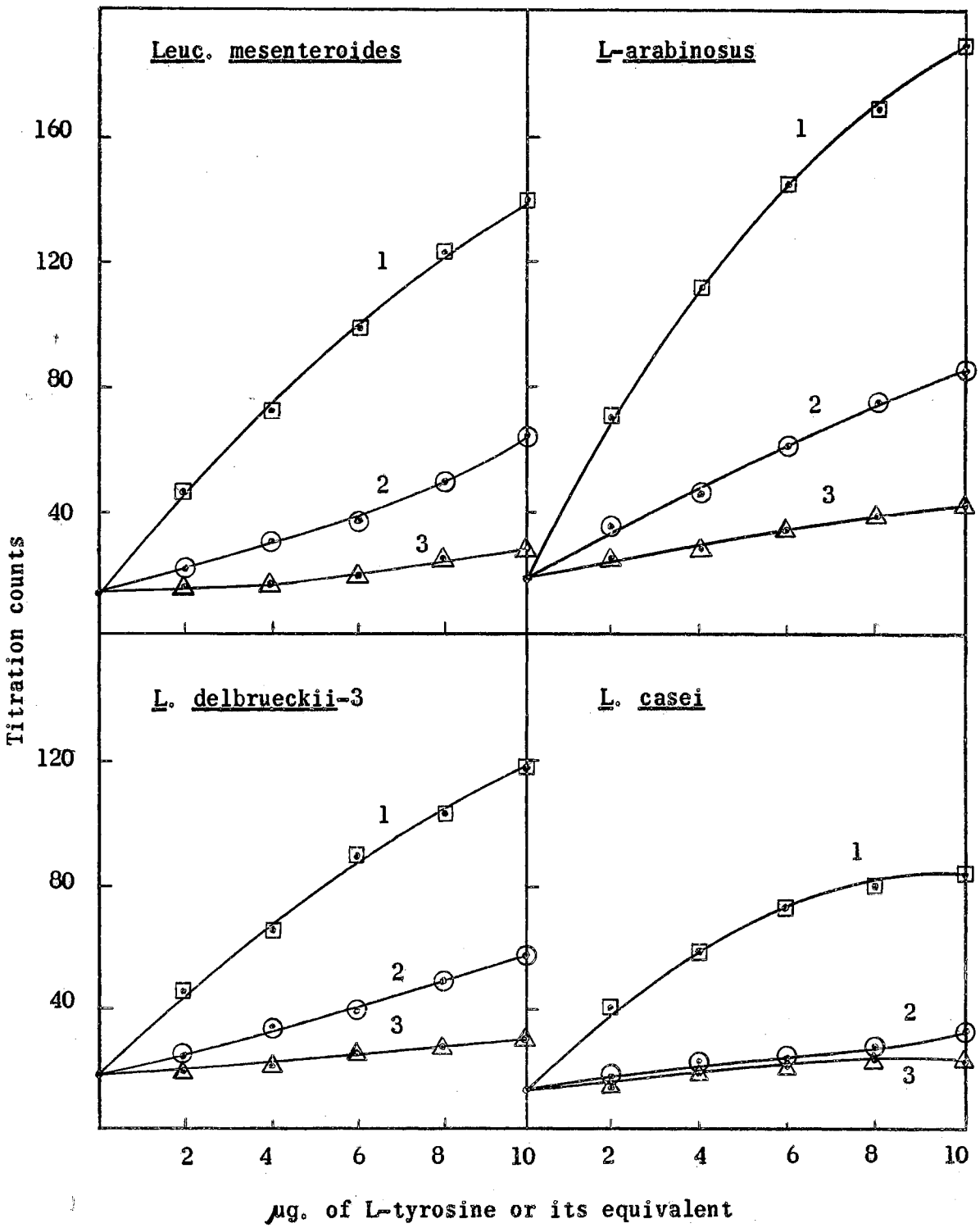


(1) Response to L-leucine

(2) Response to L-leucine in D-arabonyl-L-leucine

Figure 2

Utilization of L-Tyrosine and Glyconyl-L-Tyrosine



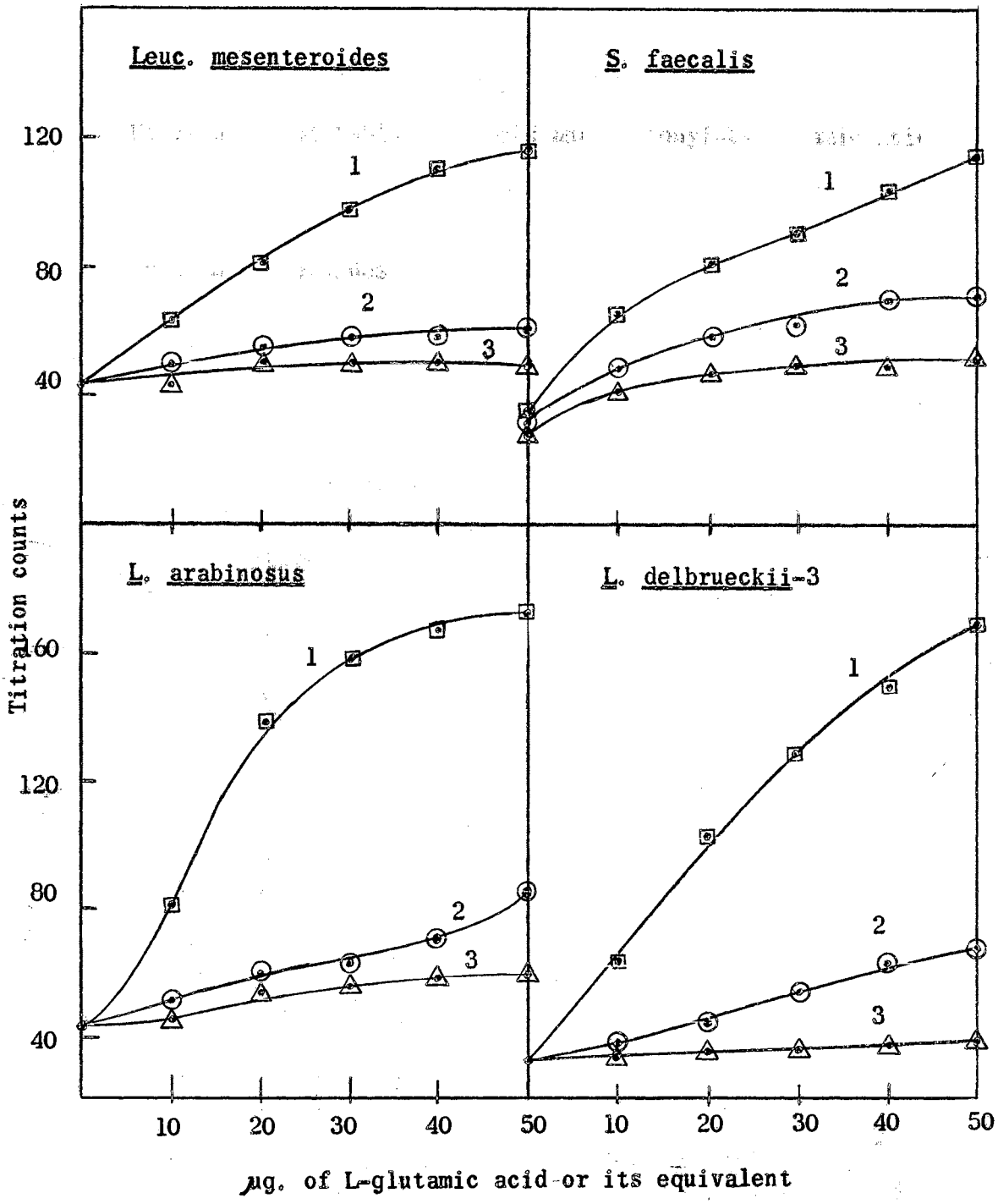
(1) Response to L-tyrosine

(2) Response to L-tyrosine in D-galactonyl-L-tyrosine

(3) Response to L-tyrosine in D-arabonyl-L-tyrosine

Figure 3

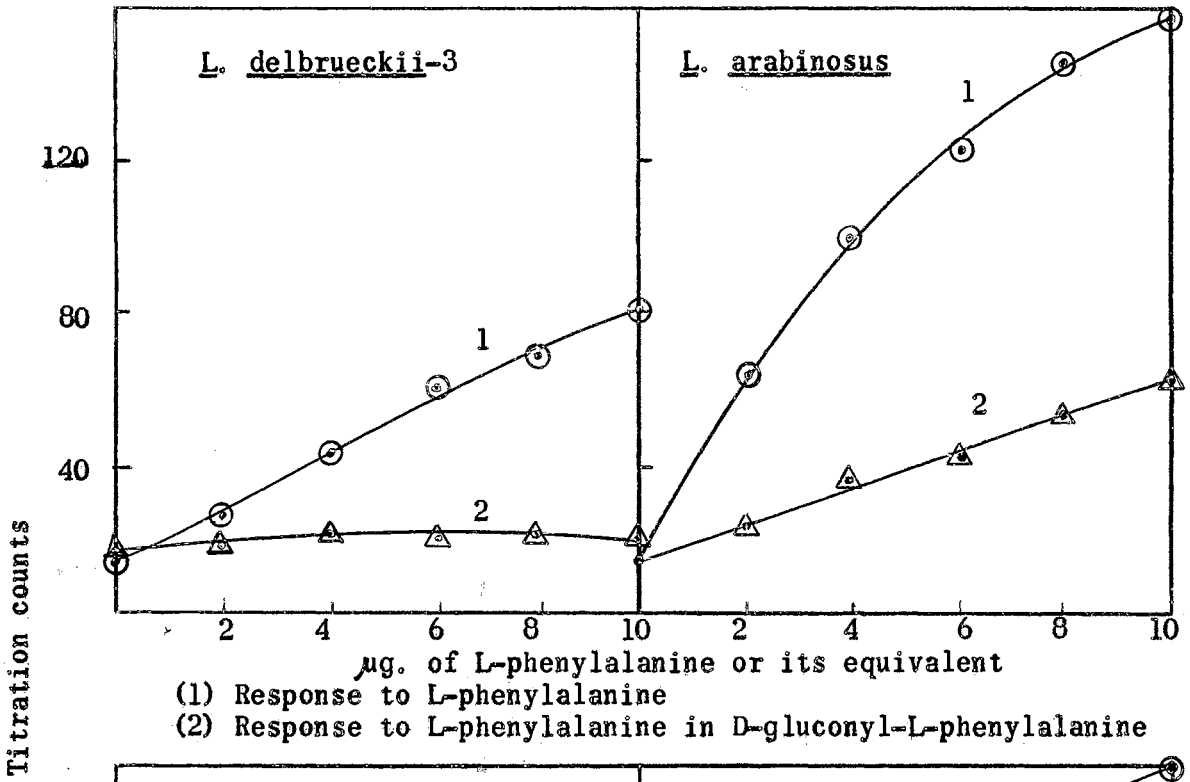
Utilization of L-Glutamic Acid and Glyconyl-L-Glutamic Acid



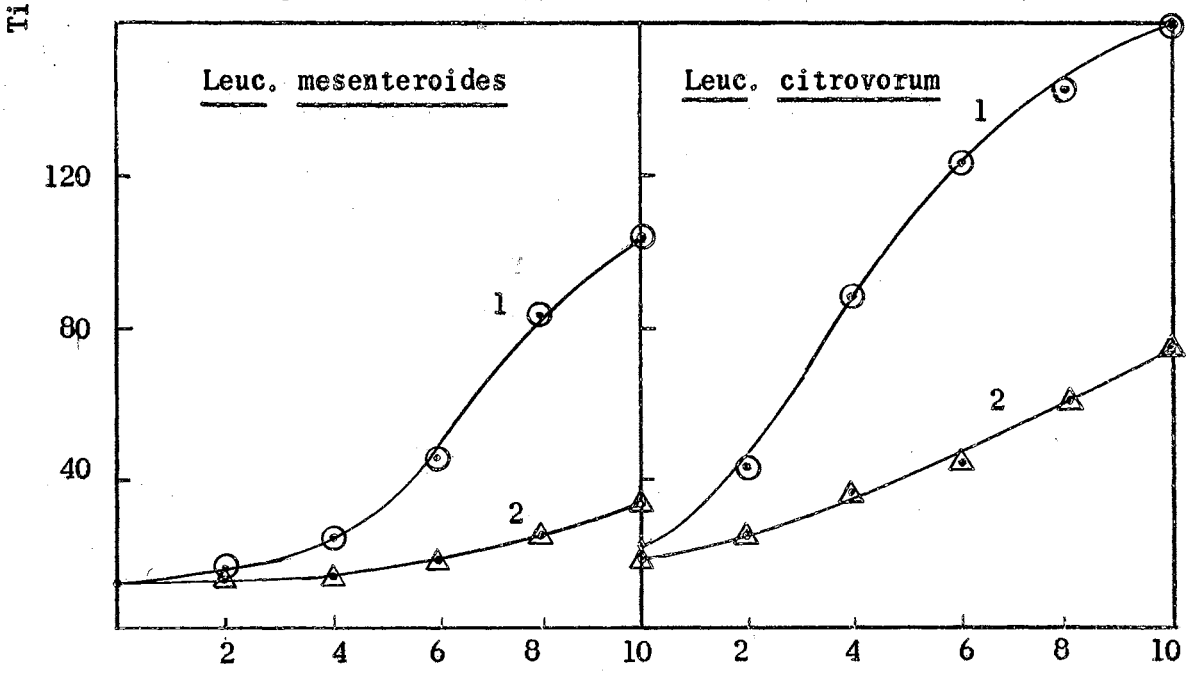
(1) Response to L-glutamic acid
(2) Response to L-glutamic in D-gluconyl-L-glutamic acid
(3) Response to L-glutamic in D-arabonyl-L-glutamic acid

Figure 4

Utilization of Free and Glyconyl-Bound L-Phenylalanine and Glycine



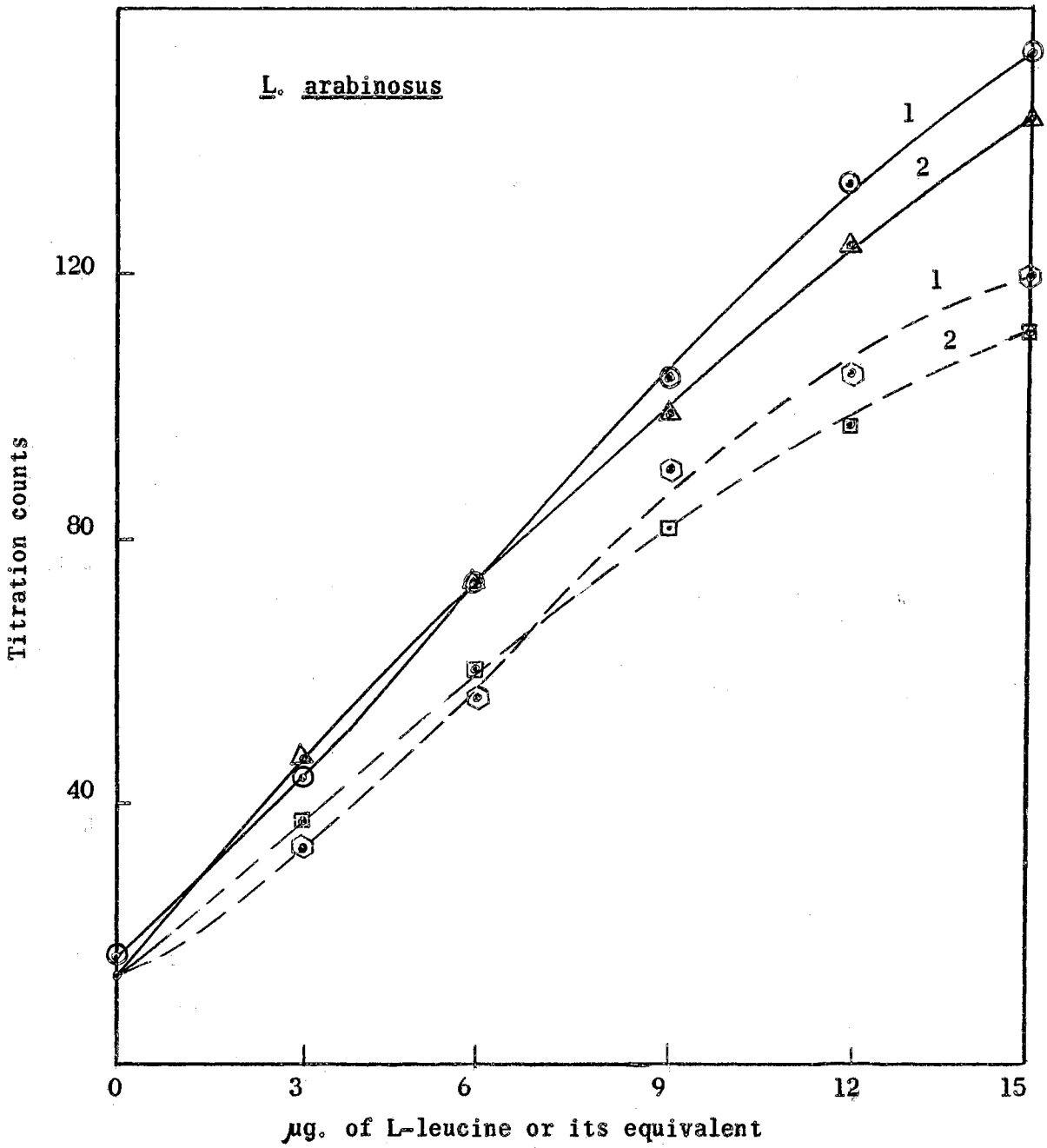
(1) Response to L-phenylalanine
(2) Response to L-phenylalanine in D-gluconyl-L-phenylalanine



(1) Response to glycine
(2) Response to glycine in D-gluconylglycine

Figure 5

The Effect of Autoclaving on the Utilization of L-Leucine and D-Arabonyl-L-Leucine



---- D- arabonyl-L-leucine
____ L-leucine
(1) Growth-limiting amino acid added aseptically
(2) Growth-limiting amino acid autoclaved with other constituents of the medium

The Utilization of Growth-limiting L-Tyrosine with Varying DL-Phenylalanine

Figure 6

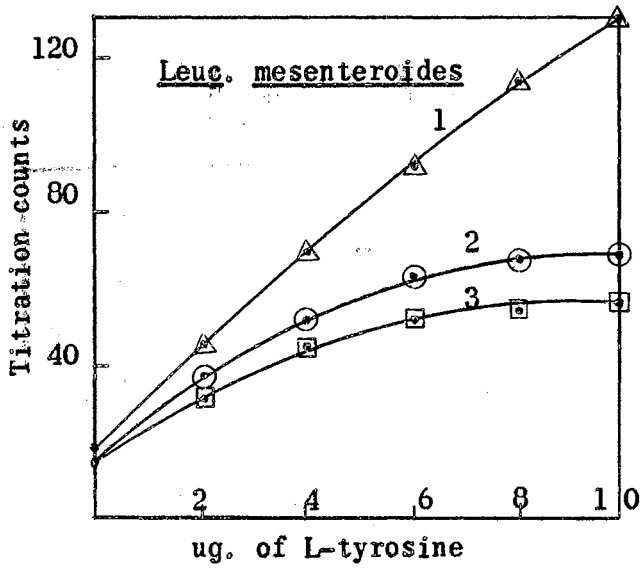
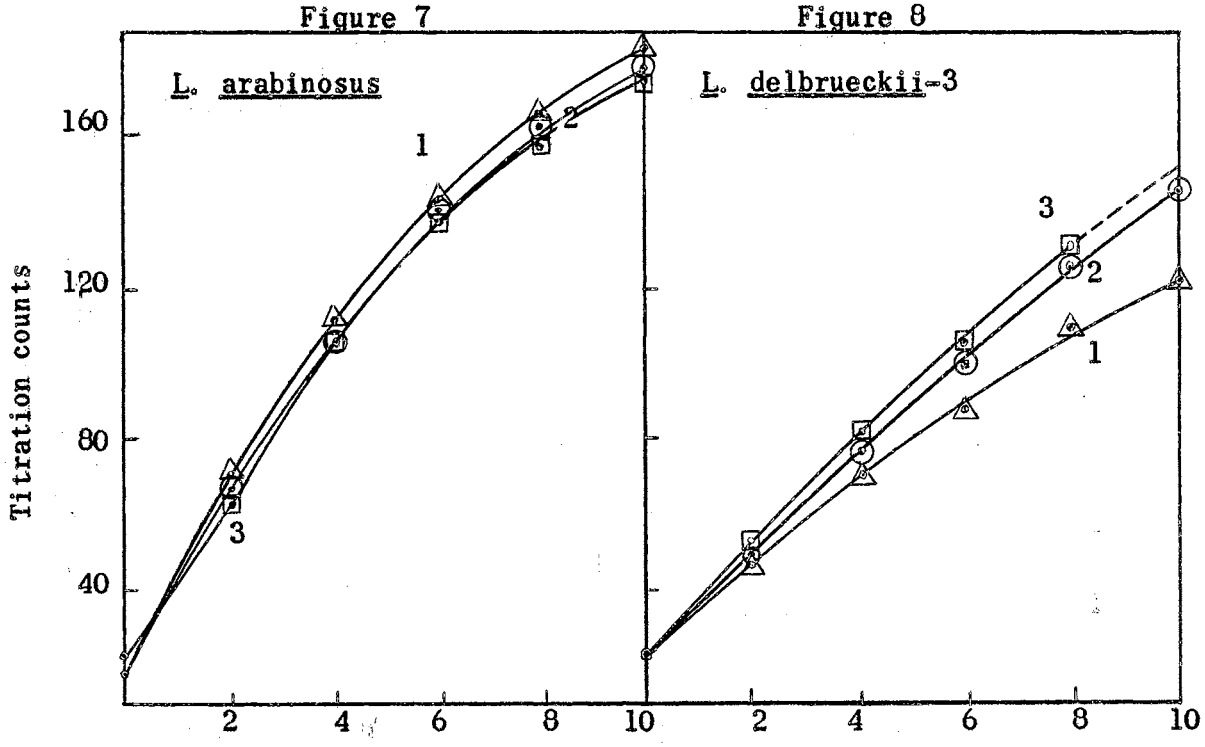


Figure 7

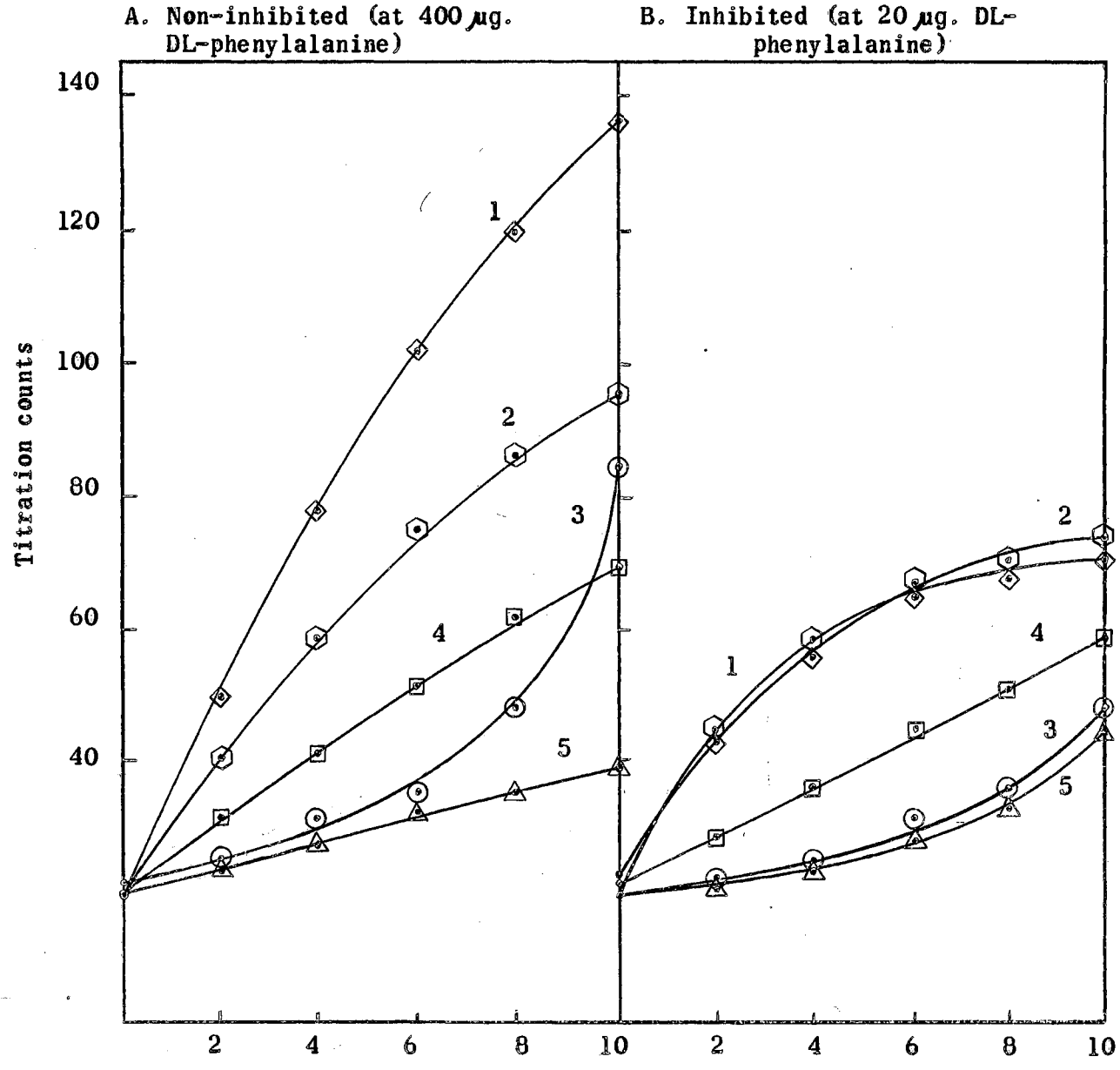
Figure 8



(1) Response at 400 ug DL-phenylalanine
(2) Response at 40 ug. DL-phenylalanine
(3) Response at 20 ug. DL-phenylalanine

Figure 9

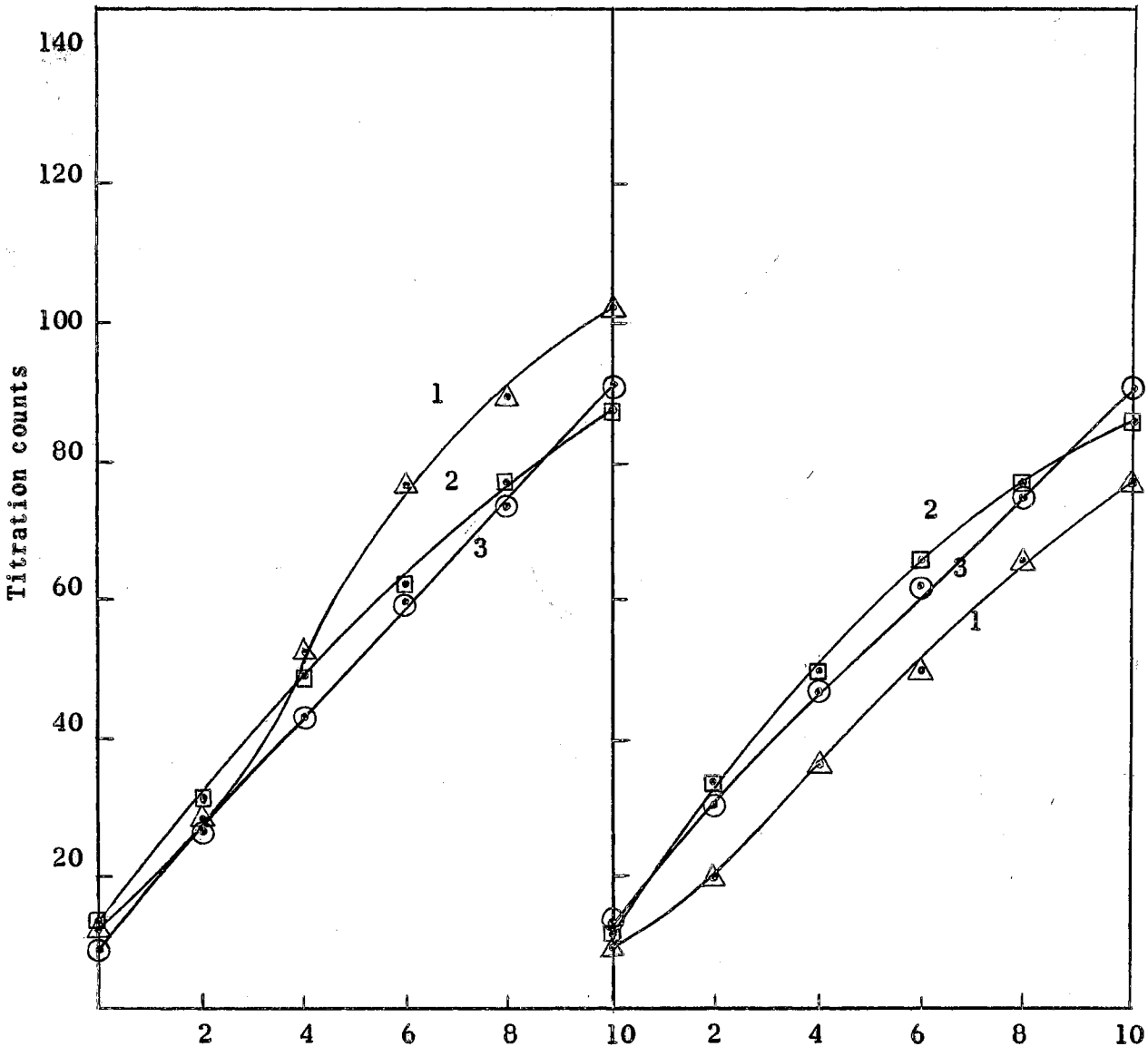
The Response of Leuc. mesenteroides to Free and Peptide-Bound l-Tyrosine under Non-inhibited and Inhibited Conditions



(1) Response to L-tyrosine
(2) Response to L-leucyl-L-tyrosine
(3) Response to glycyl-L-tyrosine
(4) Response to D-galactonyl-L-tyrosine
(5) Response to D-arabonyl-L-tyrosine

Figure 10

The Response of L. delbrueckii-3 to Free and Peptide-Bound L-Tyrosine under Non-inhibited and Inhibited Conditions



µg. of L.tyrosine or its equivalent

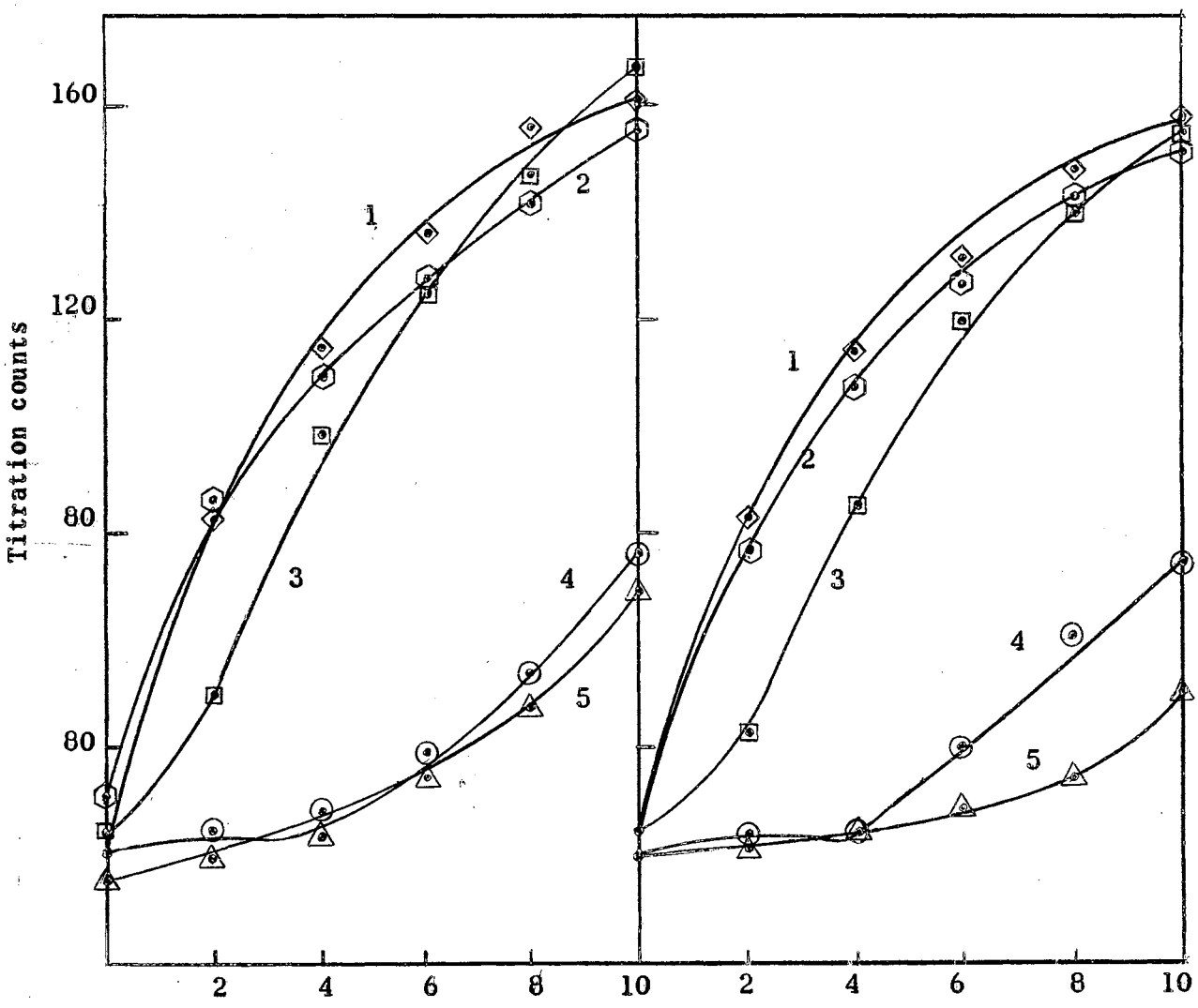
(1) Response to L-tyrosine
(2) Response to L-leucyl-L-tyrosine
(3) Response to glycyl-L-tyrosine

Figure 11

The Response of Leuc. citrovorum to Free and Peptide-Bound Glycine under Non-inhibited and Inhibited Conditions

A. Non-inhibited (at 20 μg. L-leucine)

B. Inhibited (at 200 μg. L-eucine)



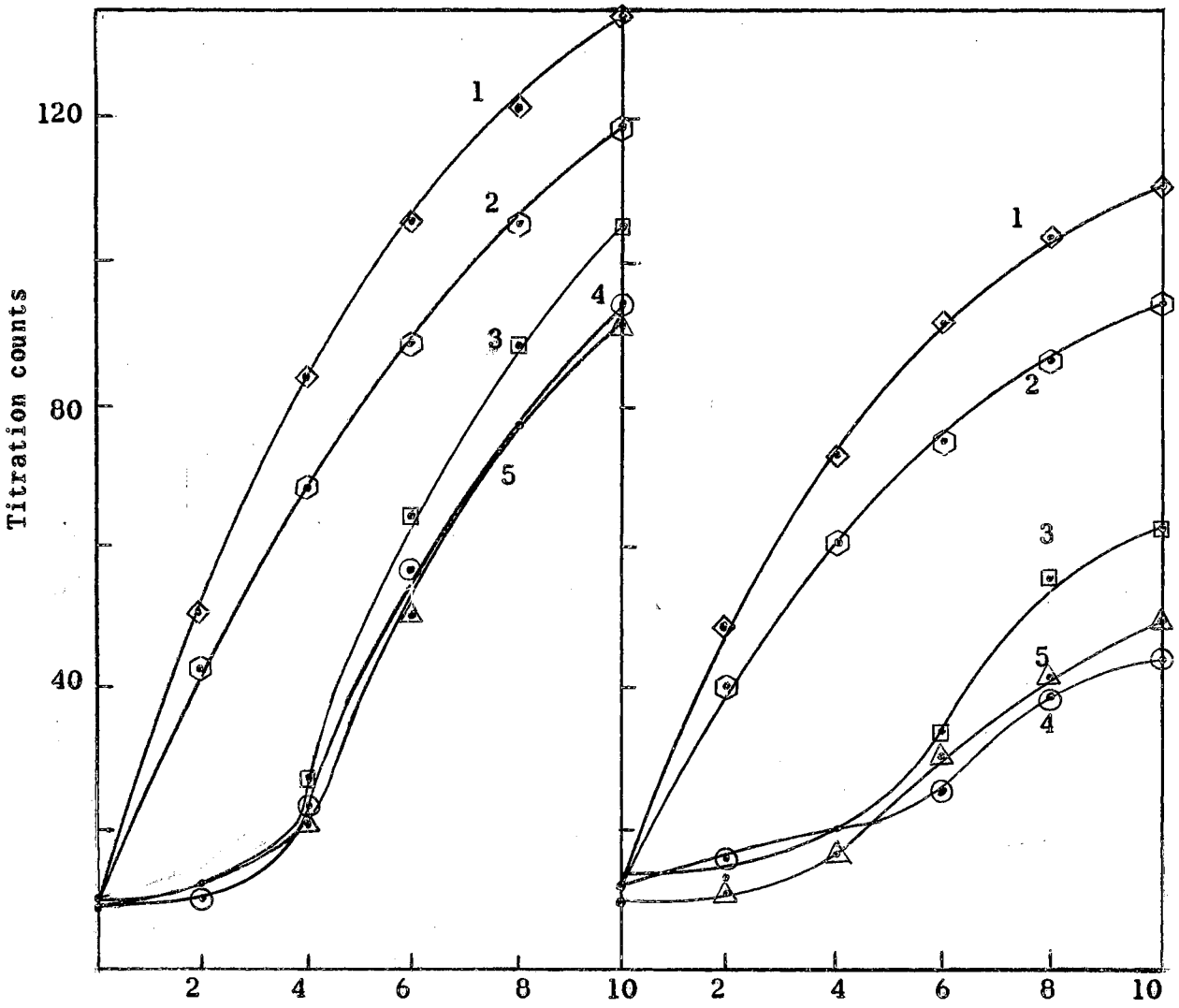
μg of glycine or its equivalent

(1) Response to glycyl-L-leucine
(2) Response to L-leucyl glycine
(3) Response to glycine
(4) Response to D-gluconyl glycine
(5) Response to D-arabonyl glycine

Figure 12

The Response of Leuc. mesenteroides to Free and Peptide-Bound Glycine under Non-inhibited and Inhibited Conditions at pH 7.0

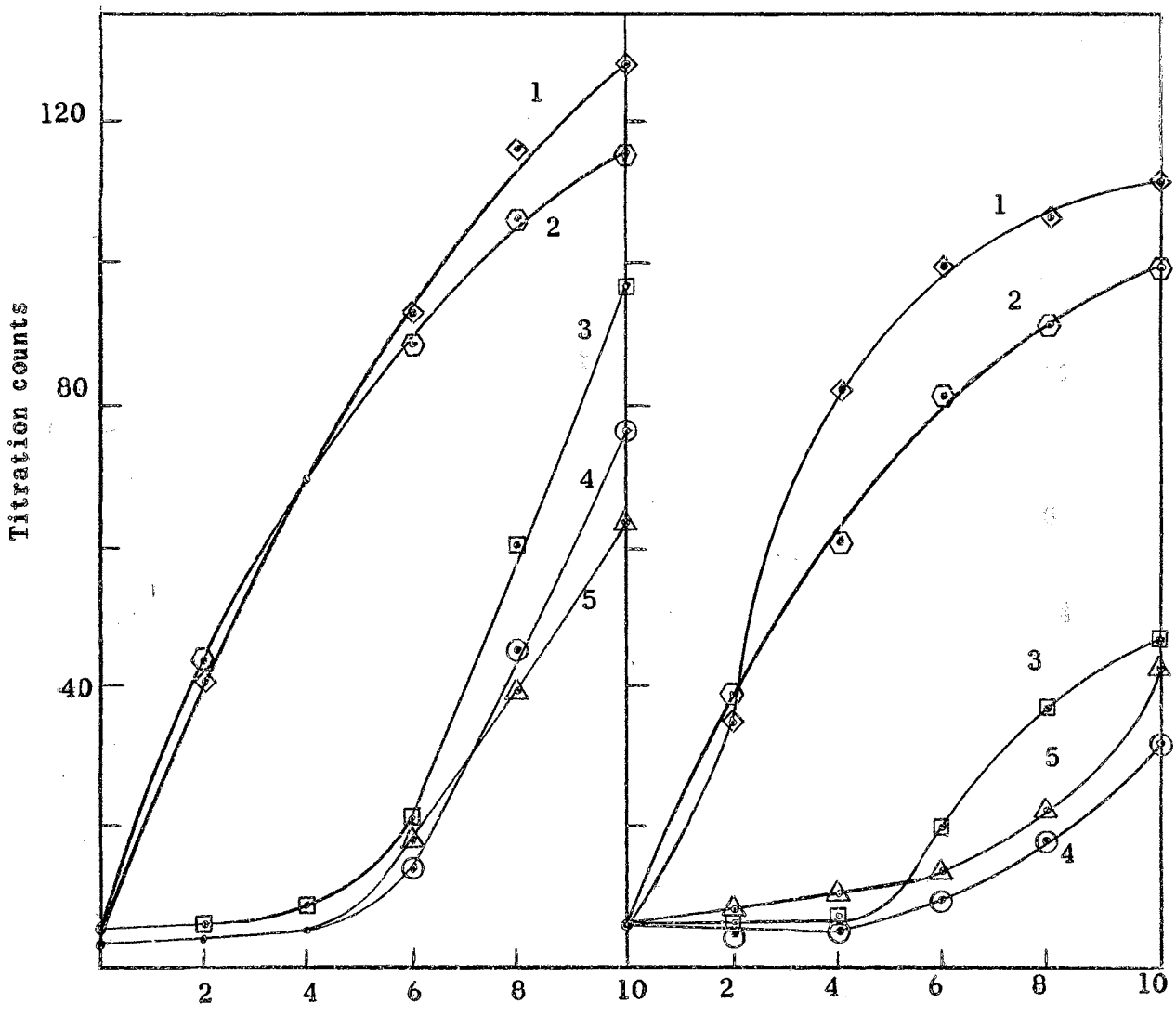
A. Non-inhibited (at 400 µg. L-arginine) B. Inhibited (at 20 µg. L-arginine)



(1) Response to glycyl-L-leucine
(2) Response to L-leucyl glycine
(3) Response to glycine
(4) Response to D-gluconyl glycine
(5) Response to D-arabonylglycine

Figure 13

The Response of Leuc. mesenteroides to Free and Peptide-Bound Glycine under Non-inhibited and Inhibited Conditions at pH 7.5

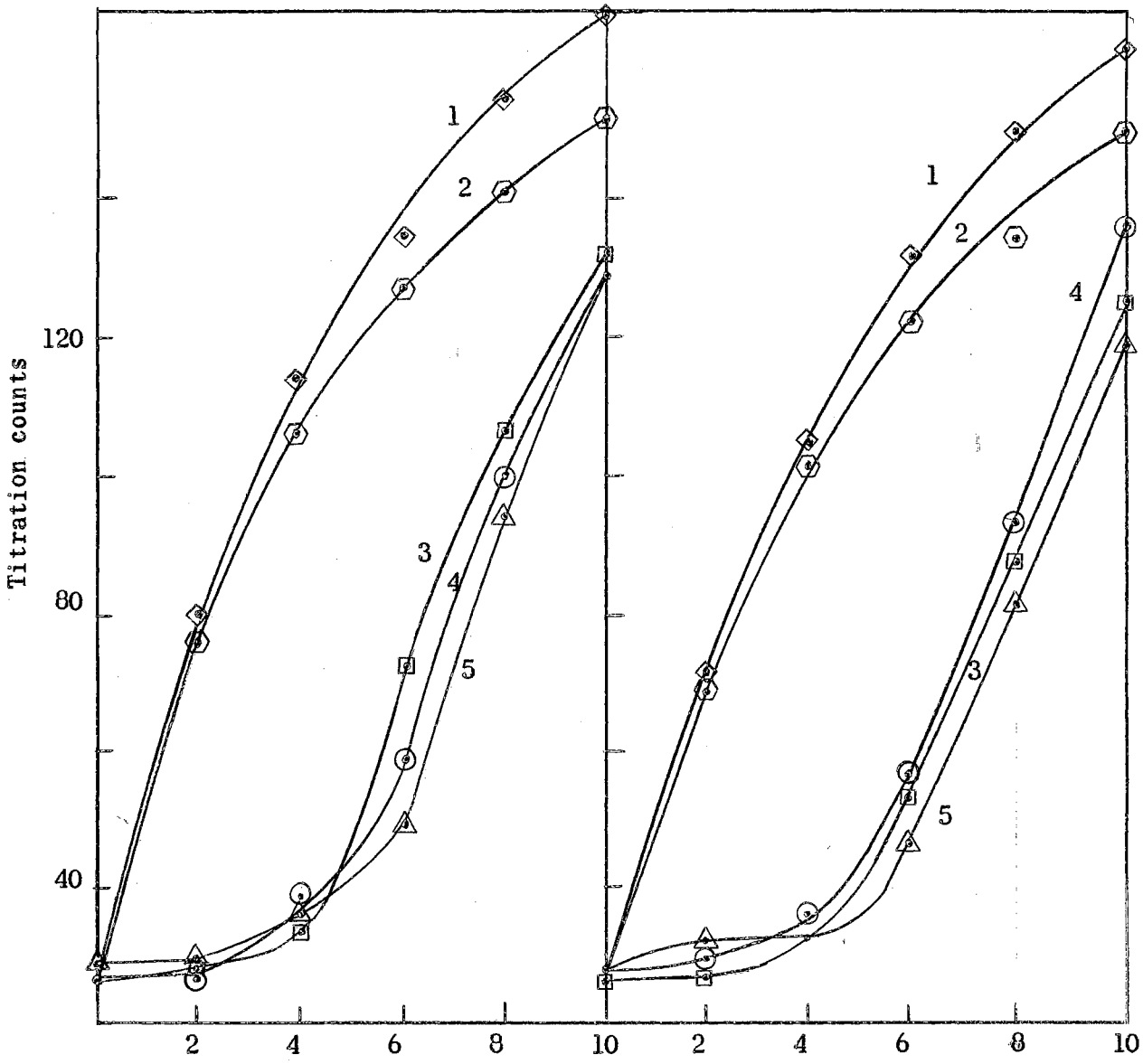


(1) Response to glycyl-L-leucine
(2) Response to L-leucyl glycine
(3) Response to glycine
(4) Response to D-gluconyl glycine
(5) Response to D-arabonyl glycine

Figure 14

The Utilization of Free and Peptide-Bound Glycine by *Leuc. mesenteroides* with Varying L-Cystine

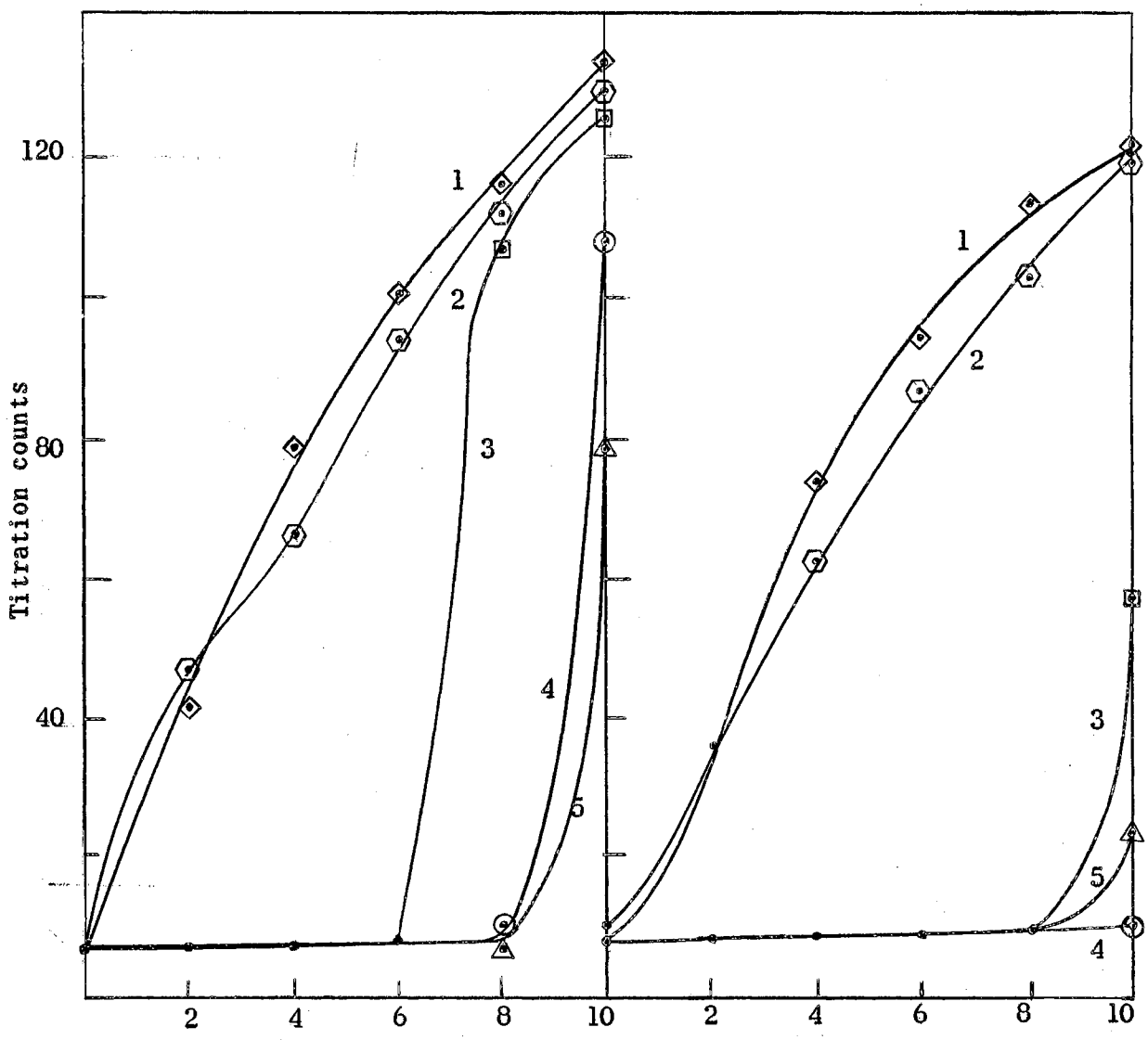
A. At 10 μg. L-cystine B. At 200 μg. L-cystine



(1) Response to glycyl L-leucine
(2) Response to L-leucyl glycine
(3) Response to D-arabonyl glycine
(4) Response to D-gluconyl glycine
(5) Response to glycine

Figure 15

The Utilization of Free and Peptide-Bound Glycine by *Leuc. citrovorum* with Varying L-Cystine



µg. of glycine or its equivalent

(1) Response to glycyl -L-leucine
(2) Response to L-leucyl glycine
(3) Response to glycine
(4) Response to D-gluconyl glycine
(5) Response to D-arabonyl glycine

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 mh.
Niacin	25.0 mg.
Ca-panto-thenate	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

Bee Tuan Uy

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