STUDIES ON THE NUTRITIONAL REQUIREMENTS OF *BACILLUS CEREUS* NRRL 569

By

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OF BACILLUS CEREUS NRRL 569

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CHAPTER I

INTRODUCTION

Microorganisms in nature require different types of nutrients in order to develop and grow. Among these different nutrients are carbohydrates, which supply both the energy and the organic carbon which the organisms need; amino acids, which act as building blocks in the formation of proteins and other compounds; vitamins, which function in coenzyme systems; and inorganic ions which serve as activators and cofactors for the enzyme systems of the organisms.

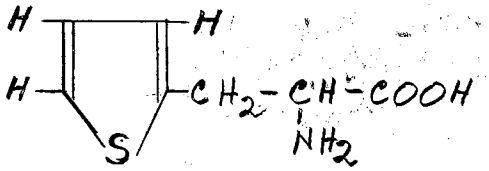
Some amino acids can be synthesized in the presence of a nitrogen and carbon source in the body of the microorganisms because they have the enzyme systems required, but some others can not be synthesized. Those amino acids which are not synthesized are sometimes called essential or basic amino acids. This is one of the objectives of this thesis, to determine the essential amino acids for Bacillus cereus (569).

The purpose of determining the essential amino acids for vegetative cells and spores of B. cereus (569) was to find a chemically defined minimal medium in which this microorganism could normally grow. As the growth was measured turbidimetrically in a Klett Photoelectric Colorimeter at 540 m μ, a normal growth was defined as that turbidity of 80 to 100 Klett Units (K. U.) resulting after incubating B. cereus (569) at 37°C for 18 hours.

There are many examples of compounds which exhibit a certain

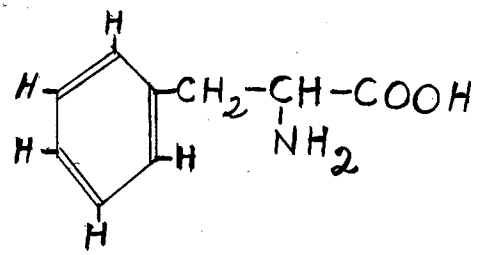
structural similarity to certain amino acids, differing from them only by one atom in the molecule which has been replaced by another atom of another functional group. The same situation exists with vitamins and hormones. In the particular case of the amino acids such compounds are called amino acid analogs, and some have the power of inhibiting the growth of an organism in absence of the corresponding metabolite. The effect of some of these amino acid analogs upon the growth of B. cereus (569) was determined in the course of this work. The structures of the analogs tested and their corresponding metabolites are given below.

Analog

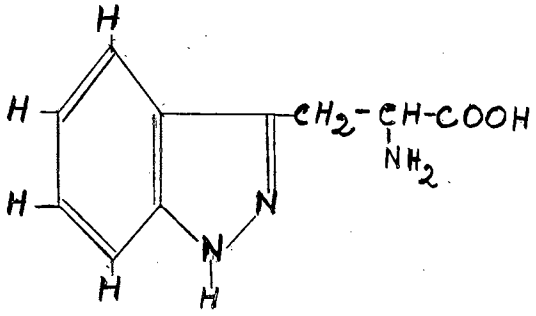


β-2-Thienylalanine

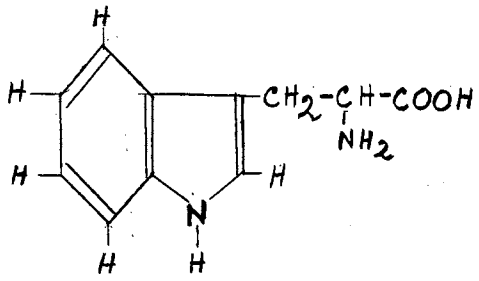
Metabolite



Phenylalanine



Tryptazan



Tryptophan

CHAPTER II

LITERATURE REVIEW

Bacillus cereus, a Gram-positive bacterium, is widely distributed and outnumbers any other spore-former found in the soil of the eastern United States. *B. cereus* is an excellent example of a stable "parent" species, having several varieties and variants (1).

Studies on the nutrition of strains of certain species of *Bacillus* show about the same uniformity of nutritional patterns within species. Such studies showed, for example, that the nutritional pattern of *B. anthracis* was different from that of the parent species *B. cereus*, since *B. anthracis* requires thiamine and is more exacting in its amino acid requirements. This fact might be used to support the classification of *B. anthracis* as a separate species rather than a variety of *B. cereus* (2).

Williams and Harper (3) tested several amino acids and found that none of them alone would serve as a source of either carbon or nitrogen, or both carbon and nitrogen, for either test strain of *B. cereus*. Growth occurred with each strain except when valine was omitted. It was found that *B. mycoides* and *B. cereus* develop poorly in ammonium salts and need amino acids in the medium. *B. subtilis* and *B. mesentericus* develop in all forms of N and show greater fermenting ability with respect to carbohydrates than that shown by *B. cereus* and *B. mycoides*. These bacteria develop only in neutral or weakly basic media (4).

Campbell and Williams (5) found that as the incubation temperature

is increased, there is an increase in the growth requirements of B. stearothermophilus, B. coagulans, and B. globigii. It is supposed that at higher temperature the enzyme responsible for the synthesis of a particular metabolite undergoes thermal inactivation and thus the organism requires an exogenous source of this compound before growth can take place. McElroy and Mitchell (6) postulated that in the case of Neurospora this microorganism may normally possess two pathways for the synthesis of essential metabolites, each having a different environmental optimum, but they also pointed out that an increase in the incubation temperature does not always result in an increase in the growth requirements.

Sporogenesis is a strictly endogenous mechanism. For spore formation to occur there must be: (a) an intracellular supply of energy, and (b) an intracellular supply or pool of nitrogenous reserves which furnish precursors of the spore material. The energy supply is the driving force for synthesis of spore constituents from their nitrogenous precursors, which are probably low molecular weight substances such as amino acids, peptides, purines, pyrimidines, nucleotides, et cetera. The events of sporogenesis suggest that this process may be an adaptive protein synthesis analogous to adaptive enzyme synthesis (7). Bacterial spores are more resistant to most killing agents than vegetative forms of the same species, but it is not clear whether this difference is due to the fact that important cellular constituents may have assumed a more stable form or whether they are being protected by a physical rearrangement or by an impervious layer (8).

There is a difference in nutritional requirements for the spores and vegetative cells of B. cereus var. terminalis; leucine was essential

for spore growth but not for vegetative cell growth. The spore and vegetative cell growth of this variety of B. cereus was slightly inhibited by adenosine and not affected by alanine. The spores required isoleucine, leucine, valine and methionine at 35°C., but the vegetative cells did not require leucine (9). Schmidt (10), working with different species of the genus Bacillus, found that biotin, glutamic acid and all the amino acids which are known to be easily converted into glutamic acid gave longer survival times of heat-treated spores. The preheating seems to lower the minimal temperature at which germination can occur. It was established early (11) that L-alanine greatly stimulates the germination of spores of the genus Bacillus. This effect of L-alanine was strongly inhibited by D-alanine in the species tested. In this way it was found that germination of B. anthracis spores was stimulated by L-alanine, L-tyrosine, and adenosine. Levinson and Hyatt (12) pointed out that the stimulatory effect which manganese has upon spore germination in B. megaterium might be explained by assuming that manganese activates a proteolytic enxyme in the spores. As a result of its proteolytic activity the enzyme in turn makes free L-alanine available for the germination process.

Working with amino acid analogs and B. cereus, Nakada, Matsushiro, and Miwatani (13) prevented the developmental stages of germination, vegetative cell growth, and re-sporulation in water. A specific reversal was obtained by the addition of the corresponding metabolites.

Instances of antagonism between two metabolites have been known for a number of years, particularly among the vitamins and amino acids. This type of study is frequently used in revealing the mechanism of various biosynthetic reactions. It has also been suggested that such antagonisms

between metabolites may comprise one of the normal regulatory mechanisms of living cells. Most of the antagonistic effects among metabolites have been observed as reversible growth inhibitions of microorganisms in minimal media (14). Presumably, most metabolite antagonists act in living cells by the inhibition of specific enzyme systems. In many instances, however, the enzymes inhibited are unknown. Studies with isolated systems, where possible, are also likely to be of considerable value in determining the mechanism of action of drugs (14). Instances in which antagonists are metabolized by living cells to less complex substances have been reported from time to time. In some other cases the antagonist is converted to a more complex compound analogous to those formed from the normal substrate (14).

The surest indication that the antagonistic effect produced by a structural analog is due to the competition of that analog with its related metabolite is the ability of the latter to reverse the action of the agent. The evidence is stronger when, over a considerable range of concentrations, a constant ratio (inhibition index) is obtained between the amounts of the two related compounds which just counterbalance each other (15).

Of the various hypotheses proposed to explain why structurally similar compounds should compete with each other in biological systems, the one which fits the observed facts best and which has been most stimulating for further discovery is the one which presents metabolite and inhibitory analog as contending for a specific part of a protein, possibly an enzyme, with which the metabolite normally reacts. The union of metabolite, or of analog, with the protein is usually a reversible one, so that relative concentrations and relative affinities of metabolite and

analog determine which one shall complex with the protein (15).

The action of certain agents is antagonized not only by the metabolites to which they show analogy, but also by structurally-related substances. Two compounds may be antagonistic because they react with each other to form precipitates or biologically inert substances (15). It is not necessary that one of a pair of antagonistic structural analogs be a metabolite in order that biological competition may be manifested. Two substances entirely foreign to an organism may be shown to contend within it in order to exert a pharmacological action (15).

In living cells, the interpretation of the action of antagonists is further complicated by the many interrelationships of enzyme systems as well as other factors, such as the penetration of cell walls (14). It is generally admitted that one mechanism for the inhibition of microbial multiplication by structural amino acid analogs is due to a blocking of protein synthesis. Studies with analogs also strongly suggest that "abnormal" amino acids, closely related in structure to the naturally occuring molecules, can substitute for the latter in the fabric of proteins (16). The toxicity of the analog is then based on the synthesis of structurally and functionally abnormal protein containing it. Munier and Cohen (17) thus support the idea that under conditions where there is an inhibition of cellular multiplication, the presence of structural amino acid analogs results not in a blocking of protein synthesis, but in the synthesis of proteins which are abnormal in their structure and in their activity.

The nature of the work to be covered in this thesis is basically to lay the nutritional groundwork for a study of the effect of some selected amino acid analogs on Bacillus cereus NRRL 569. B. cereus (569) is capa-

ble of producing adaptively the enzyme penicillinase. A long-range project in this laboratory is a study of the incorporation of amino acid analogs into the penicillinase, and other cellular proteins, of B. cereus sp.

In order to study and interpret properly the metabolism and effect of amino acid analogs in any organism, one must first know the amino acid requirements of that organism. In addition, a knowledge of any other essential nutritional requirements can be most helpful. Therefore, the basic problem of this thesis is to define a minimal chemical medium which will allow a significant growth response for the organism, B. cereus (569).

CHAPTER III

EXPERIMENTAL

The microorganism used in this study was Bacillus cereus NRRL 569 which has the ATCC number 10876. To insure the purity of the culture, this strain of B. cereus was periodically reisolated by the cross streak-plate method in our laboratory. After the reisolation, the bacterium was kept on agar slants. Every four weeks, five sterile agar slants were numerated and then inoculated, using a nichrome loop previously flamed in a Bunsen burner; then the slants were incubated at 37°C. for 24 hours, and after this period of time, the slants were stored in the refrigerator. Slant No. 1 was used in all the inoculations carried out during the first week, slant No. 2 the second week, et cetera. At the end of the fourth week, slant No. 5 was used to inoculate another five agar slants and the procedure was repeated each month.

Graph of Klett Readings vs. Dry Weight Cells: For this experiment, a casamino acid hydrolyzate, prepared by the method of Muller (18), was used as the nitrogen source. This hydrolyzate contained 5 gr. of total amino acid per 100 ml. of solution. The complete medium contains in addition to the casamino acid hydrolyzate, mineral salts as shown below in Table 1.

The pH was adjusted to 7.3 and the total volume was made up to 1000 ml. with distilled water. The medium was then placed in a 6 liter Erlenmeyer flask provided with a cotton plug and autoclaved at 15 pounds for

15 minutes, and incubated at 37°C. for 5 days. At the same time, two 50 ml. Erlenmeyer flasks containing 15 ml. of the medium were treated in the same way. One of these 50 ml. flasks was inoculated with B. cereus from a nutrient agar slant, and incubated on a shaker at 37°C. for 20 hours. The entire contents of this 50 ml. flask were used in the inoculation of the 1000 ml. of medium in the 6 liter Erlenmyer flask. This 1000 ml. of casamino acid medium, after being inoculated, was incubated on a gyratory shaker at room temperature for 14.5 hours. At the end of this period, the growth was stopped by the addition of a large amount of Na azide.

Table I

COMPOSITION OF THE MEDIUM

Casamino acid hydrolyzate (5%)	400 ml.
KH_2PO_4	6.5 gm.
$MgSO_4 . 7H_2O$	0.4 gm.
0.1% $FeSO_4$ solution	1.0 ml.

As shown in the table below, a series of dilutions was then made, using sterile casamino acid medium as the diluting agent. The contents of the second 50 ml. Erlenmeyer flask prepared above were used as the blank. The turbidity measurements were made in a Klett-Summerson Photoelectric Colorimeter at 540 mμ (green filter).

After the readings were taken, each of the dilutions prepared below were centrifuged; the supernatants were carefully decanted, and the residues resuspended in double-distilled water and centrifuged again. The

washing was repeated three times. Finally, the residue in each centrifuge bottle was resuspended in a few ml. of water and transferred onto previously tared aluminum plates by means of a 10 ml. serological pipette, and dried in an oven at 110°C. for three hours. The difference between the weight of the aluminum plates before and after plating the samples gave the weight of the dry cells. All the weighing was done on a Mettler Gram-atic balance. The results are summarized in both Table II and Figure 1.

Table II

GROWTH VS. DRY WEIGHT OF CELLS

Ml. of Suspension	Ml. of Pure Medium	Klett Units	Wt. of the Sample
0	100	0	-
10	90	46	10.45 mgs.
20	80	74	18.10 mgs.
30	70	105	24.10 mgs.
40	60	127	31.00 mgs.
50	50	148	37.80 mgs.
60	40	168	45.45 mgs.
70	30	188	47.75 mgs.

Utilization of Amino Acids as the Sole Source of Carbon: In order to determine how efficiently the amino acids would serve as an energy source, the following experiment was devised. The growth of vegetative cells in a complete medium, containing glucose and citrate, in addition to amino acids, was compared with growth in a medium lacking the glucose

The turbidity was measured at 540 mμ (green filter)

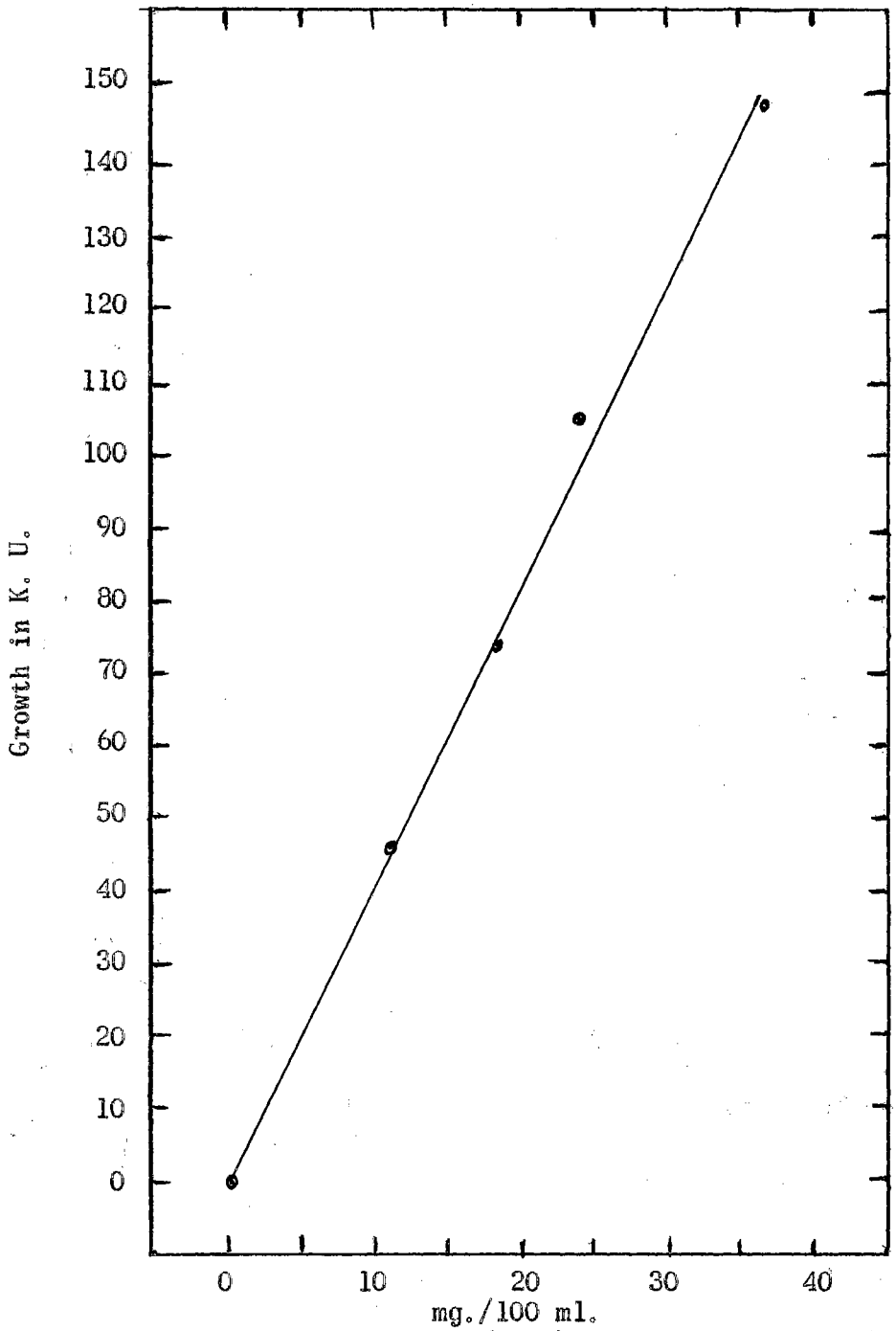


Figure 1. Plot of growth (K.U.) vs. dry weight of cells per 100 ml.

and citrate. In addition, the possible effect of two B vitamins was checked by use of a third complete medium containing added biotin and pantothenate.

The composition of each medium, exclusive of the 18 amino acids which were present in all three, is given below.

Table III

COMPOSITION OF THE NON-AMINO ACID MEDIA
GLUCOSE, CITRATE, NON-AMINO ACID MEDIUM

Medium I	
Glucose (its final concentration in the medium was 1%)	1.60 gm.
Sodium Citrate	0.32 gm.
NH_4Cl	0.32 gm.
0.16% $(NH_4)_2Fe(SO_4)_2$	0.96 ml.
$MgSO_4 \cdot 7H_2O$	0.048 gm.
KH_2PO_4	0.8704 gm.
Distilled H_2O	to make 100 ml.

Table IV

GLUCOSE-FREE, NON-AMINO ACID MEDIUM

Medium II	
NH_4Cl	0.32 gm.
0.16% $(NH_4)_2Fe(SO_4)_2$	0.96 ml.
$MgSO_4 \cdot 7H_2O$	0.048gm.
KH_2PO_4	0.8704 gm.
Distilled H_2O	to make 100 ml.

Vitamin-Fortified Non-Amino Acid Medium (Medium III): This medium was simply medium I plus traces of biotin and pantothenic acid.

Five ml. of the non-amino acid media I, II, and III were transferred into the corresponding culture tubes, after the pH was adjusted to 7.2 with concentrated NaOH. To each of the culture tubes containing the non-amino acid medium, 2 ml. of a solution containing 18 amino acids was added. As the amino acid solution used had a concentration of 800 γ/ml. of each amino acid, the amino acid concentration was 1600 γ/tube. Then the pH of all the tubes was re-adjusted to 7.2 and the volume of each tube made up to 8 ml. The final amino acid concentration was then 200 γ/ml. The tubes were autoclaved at 15 pounds for 10 minutes, and incubated at 37°C. for 24 hours.

The inoculation was carried out from a 24 hour culture in nutrient broth which was first centrifuged and washed three times with sterile saline solution to prevent the transfer of any nutrient material along with the inoculation of cells. The saline suspension used for the inoculation read 130 KU. The readings were taken 21 hours after the inoculation. In the case of Medium III, the vitamins were added after the autoclaving in order to avoid their destruction by heating. The inoculation was made using five drops of the saline suspension. The results are given in Table V below.

From this data it was decided that the non-amino acid medium I would be used throughout this work. It should be pointed out that the two B vitamins tested did not stimulate growth and are thus not required by B. cereus (569).

Table V

GROWTH IN THREE DIFFERENT NON-AMINO ACID MEDIA

Non-Amino Acid Medium	K.U.
I + Azide	0
I + Azide	0
I	276
I	276
II	62
II	59
III	266
III	272

Determination of a Minimal Amino Acid Medium for Vegetative Cells: In this series of experiments, non-amino acid medium I was used as a 16-fold concentrate; so that when a 0.5 ml. portion was diluted to the final culture tube volume of 8 ml., it was of standard strength. The eighteen amino acid stock solutions used had a concentration of 4 mg./ml. of each amino acid; 0.4 ml. of these solutions was then used, which when diluted to 8 ml. gave a final concentration in the culture medium of 200 γ/ml. of each amino acid.

0.4 ml. portions of the amino acid solutions were dispensed into the corresponding culture tubes, which were previously washed with "Tide," rinsed with tap water, steam condensate and distilled water. To one of the culture tubes all the 18 amino acids were dispensed, but in each of the remaining tubes one amino acid was omitted. Also, a mixture of all the amino acids plus Na azide was prepared to be used as the blank. The

pH of every tube was adjusted to 7.2 with NaOH, and the volume made up to 8 ml. All the culture tubes were run in duplicate, and autoclaved at 15 lb. for ten minutes. After autoclaving, it was observed that the medium had different colorations due to a different degree in the caramelization of glucose. This difference in the coloration introduced a certain small error in our measurements in this experiment. In later experiments this was solved by autoclaving the glucose and the amino acids separately.

After a 24 hour incubation at 37°C., the culture tubes containing the culture media were inoculated with a fresh suspension of B. cereus in saline solution. This suspension was prepared by centrifuging and washing three times the cells grown in a 2% casamino acid broth. The washing was to eliminate any trace of amino acids adsorbed on the surface of the bacteria which might influence our results. The suspension used read 116 K. U. against saline solution as the blank, and five drops of this suspension per tube were used for the inoculation. At the end of 18 hours two drops of 5% Na azide were added to each of the culture tubes to stop the growth and the readings were taken. The data are recorded in Table VI.

From the results below, it was then decided to make another run, but using only ten amino acids. The non-amino acid solution was this time four times stronger than the original and 2 ml. of this solution was used per culture tube. This volume and concentration of the non-amino acid medium will be used throughout all this work. Its composition for one liter of the non-amino acid medium is given in Table VII.

Table VI

GROWTH OBTAINED WHEN THE ESSENTIALITY OF EIGHTEEN DIFFERENT AMINO ACIDS WAS TESTED

Amino Acid Composition	K.U.
All 18 present + azide	0
All 18 present + azide	0
All 18 present	158
All 18 present	160
Amino Acid Left Out	
DL-Tryptophan	133
DL-Tryptophan	134
DL-Threonine	153
DL-Threonine	152
L-Histidine.HCl	120
L-Histidine.HCl	125
L-Arginine.HCl	122
L-Arginine.HCl	120
L-Lysine	198
L-Lysine	202
L-Leucine	47
L-Leucine	49
DL-Isoleucine	107
DL-Isoleucine	104
DL-Methionine	214
DL-Methionine	210
DL-Valine	0
DL-Valine	0
DL-Phenylalanine	194
DL-Phenylalanine	194
L-Proline	290
L-Proline	290
L-Cysteine	296
L-Cysteine	298
DL-Serine	216
DL-Serine	212
L-Glutamic Acid	122
L-Glutamic Acid	122
DL-Aspartic Acid	186
DL-Aspartic Acid	190
L-Tyrosine	160
L-Tyrosine	160
Glycine	168
Glycine	168
DL-Alanine	172
DL-Alanine	172

Table VII

COMPOSITION OF THE NON-AMINO ACID MEDIUM

Glucose (gives a concentration of 1% in the final medium)	40 gm.
NH_4Cl (gives a concentration of 0.2% in the final medium)	8 gm.
Na Citrate (gives a concentration of 0.2% in the final medium)	8 gm.
$MgSO_4 \cdot 7H_2O$	1.2 gm.
KH_2PO_4	21.76 gm.
0.16% $(NH_4)_2Fe(SO_4)_2$	24 ml.
Distilled water to make 1000 ml.	

In order to avoid the error introduced by the caramelization of glucose observed in the previous experiment, the non-amino acid medium was autoclaved separately from the amino acid medium at 15 lbs. for five minutes, and then added aseptically to the autoclaved amino acid medium by means of a 2 ml. delivering pipette. The pH of each culture tube was, as in the experiment before, previously adjusted to 7.2. The inoculation was carried out following the same pattern as in previous experiments.

Another similar experiment, but with only five amino acids was run. This time the final concentration of the amino acids in the culture medium was increased to 400 γ/ml. of each amino acid. The same procedure described for the previous experiments was followed, but this time the same medium used in the experiment was also used to grow the inoculum, to circumvent any adaptive phenomena. This experiment was run twice, and the effect of glycine upon the growth was very well established. The five amino acids used were: DL-valine, L-leucine, L-glutamic acid, DL-isoleucine, and L-tyrosine. The data are given in Table IX.

Table VIII

GROWTH RESPONSE WHEN THE ESSENTIALITY OF TEN AMINO ACIDS WAS TESTED

Amino Acid Composition	K.U.
All 10 amino acids + azide	0
All 10 amino acids + azide	0
All 10 amino acids	161
All 10 amino acids	166
Amino Acid Left Out	
DL-Tryptophan	132
DL-Tryptophan	135
DL-Threonine	152
DL-Threonine	147
L-Histidine.HCl	132
L-Histidine.HCl	136
L-Arginine.HCl	135
L-Arginine.HCl	131
L-Leucine	43
L-Leucine	39
DL-Isoleucine	112
DL-Isoleucine	112
DL-Valine	0
DL-Valine	0
L-Glutamic Acid	107
L-Glutamic Acid	110
L-Tyrosine	121
L-Tyrosine	123
Glycine	132
Glycine	131

Table IX

GROWTH RESPONSE OBTAINED DURING THE DETERMINATION OF THE ESSENTIALITY OF FIVE AMINO ACIDS

Amino Acid Composition	K.U.
All 5 amino acids + azide	0
All 5 amino acids + azide	0
All 5 amino acids	41
All 5 amino acids	40
All 5 amino acids + glycine	69
All 5 amino acids + glycine	70
Amino Acid Left Out	
DL-Valine	0
DL-Valine	0
L-Leucine	33
L-Leucine	36
L-Glutamate	0
L-Glutamate	0
DL-Isoleucine	0
DL-Isoleucine	0
L-Tyrosine	26
L-Tyrosine	26

From the data above, it can be seen that L-leucine, and L-tyrosine have little effect on the growth of the organism; it grows fairly well compared to the control in the absence of these amino acids. Glycine showed a very strong stimulatory effect. Based upon these results it was decided to use DL-valine, L-glutamic acid, DL-isoleucine, and glycine as the four basic amino acids in further studies of the growth of B. cereus (569).

In the next experiment the concentration of each of these four amino acids in the final medium was increased up to 800 γ/ml. The effect of other amino acids upon the growth was then measured, using the growth in the four basic amino acids as the normal growth. The data are given in Table X.

Table X

GROWTH IN THE FOUR BASIC AMINO ACIDS MEDIUM, AND IN OTHER MEDIA OF DIFFERENT COMPOSITION

Amino Acids Content	K.U.
4 basic amino acids + azide	0
4 basic amino acids + azide	0
4 basic amino acids	72
4 basic amino acids	72
4 basic amino acids + L-Arginine.HCl	81
4 basic amino acids + L-Arginine.HCl	80
4 basic amino acids + L-Histidine.HCl	84
4 basic amino acids + L-Histidine.HCl	84
4 basic Amino acids + DL-Aspartate	68
4 basic amino acids + DL-Aspartate	68
4 basic + L-Arginine.HCl + L-Histidine.HCl	90
4 basic + L-Arginine.HCl + L-Histidine.HCl	90
4 basic + L-Arginine.HCl + L-Histidine.HCl + DL-Aspartate	70
4 basic + L-Arginine.HCl + L-Histidine.HCl + DL-Aspartate	70

None of the singly-tested amino acids showed a large stimulatory effect upon the growth of the bacterium. More than that, in all the cases where aspartic acid was used, there was, if anything, an inhibition of the growth.

Growth Curve for B. cereus (569) in the Four Basic Amino Acid Medium: 0.8 ml. of an 8 mg./ml. solution of the four basic amino acids (amino acid medium) were pipetted into each of twenty culture tubes and the volume was made up to 6 ml. with distilled water. The pH of the amino acid medium was previously adjusted to 7.2 with concentrated NaOH. The pH of the non-amino acid medium, prepared as in the experiments before, was adjusted to 7.2. The amino acid medium was autoclaved at 15 lb. for 10 minutes, and the non-amino acid medium for 5 minutes. Then 2 ml. of the non-amino acid medium was added aseptically to the amino acid

medium by means of a delivering pipette.

At the same time, a broth of the same composition as the medium in the culture tubes was prepared, autoclaved and inoculated from an agar slant. When the growth in the broth was of 100 K.U., the inoculation of the culture tubes was carried out by means of a sterile hypodermic syringe. The cap of each culture tube containing the medium was removed. The tube was flamed in a Bunsen burner, five drops of the inoculum were added, the tube was flamed again, and the cap returned to its original place. All the culture tubes were placed in an incubator at 37°C., keeping the inclination of each of the culture tubes constant by use of a special rack. The readings were taken by removing a pair of culture tubes every hour, stopping the growth with two drops of 5% Na azide, thoroughly agitating the tubes to get a homogeneous suspension, and then taking the readings in a Klett Photoelectric Colorimeter using the green filter. The results are summarized in Figure 2. The lag period, as determined in this experiment and for this four basic amino acid medium was of six to seven hours.

In order to check and extend the above results, two additional experiments were devised. In these runs, two different media were tested in addition to the one containing DL-isoleucine, DL-valine, L-glutamic acid, and glycine. One of these media had DL-methionine in addition to the four amino acids, for O'Brien and Campbell (9) had reported that methionine was essential for B. cereus var. terminalis. The third medium was made by replacing glycine with DL-methionine in the four basic amino acid medium. The procedure followed was the same as the one of the experiment before except the readings were taken every three hours instead of every hour for a period of 18 hours. In this case, the lag period for the three media tested was 2 to 2.5 hours. The data are summarized in Figure 3.

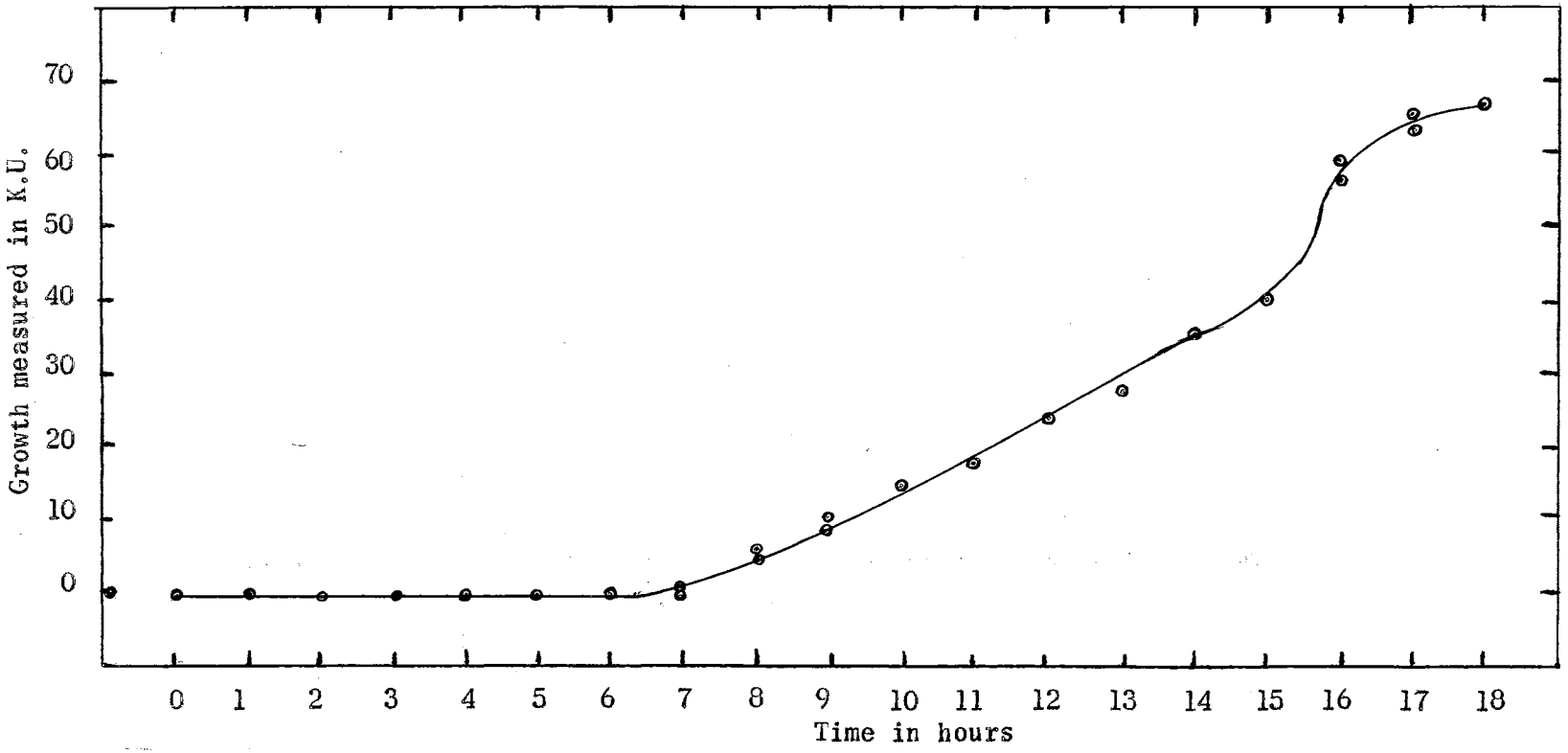


Figure 2. Growth curve of B. cereus (569) in the four basic amino acid medium

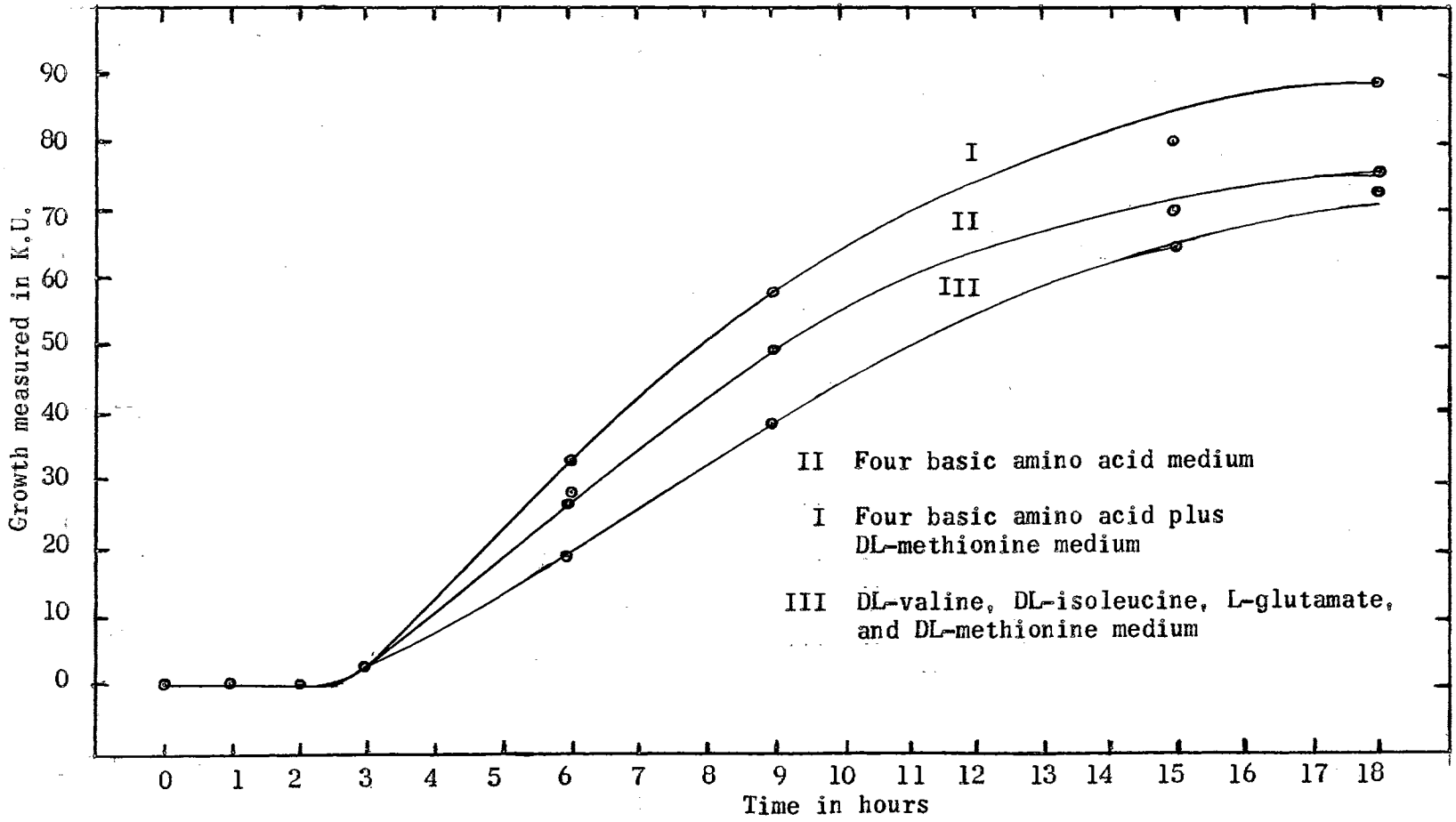


Figure 3. Growth curve of B. cereus (569) in three different amino acid media

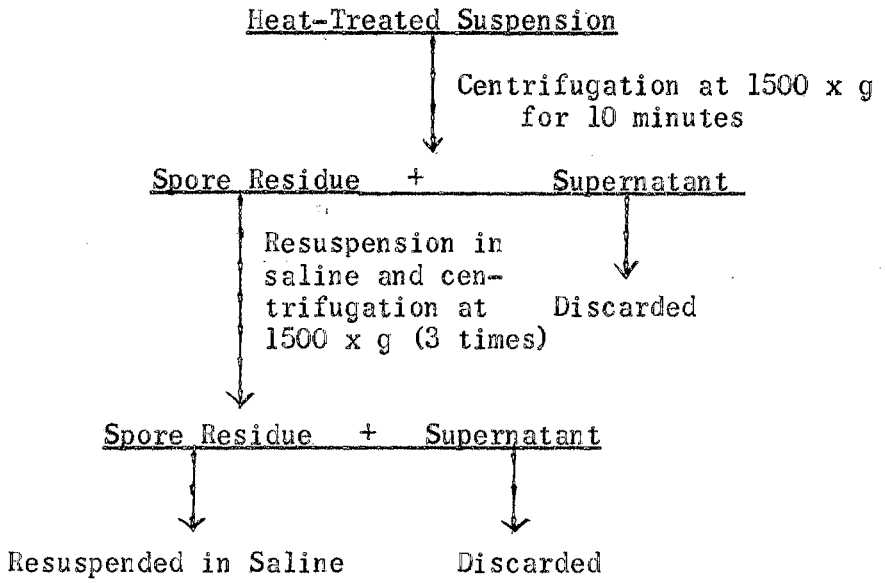
From here on it was decided to use the five basic amino acid medium (DL-valine, L-glutamic acid, glycine, DL-isoleucine, and DL-methionine) as the medium for growth studies with vegetative cells of B. cereus (569). This medium thus constitutes a minimal amino acid medium for this organism.

Determination of a Minimal Amino Acid Medium for Spore Germination and Outgrowth

Preparation of the Spores: Into five scrupulously clean 50 ml. Erlenmeyer flasks was transferred 15 ml. of Pollock's Medium S (18) for spores. The flasks were provided with cotton plugs, autoclaved at 15 pounds for ten minutes, and incubated at 37°C. for 24 hours. The inoculation was carried out with a hypodermic syringe, and a 24 hour culture grown in the five basic amino acid medium was used as inoculum. After the inoculation the five Erlenmeyer flasks were set in a Dubnoff at 37°C. with constant shaking for about 48 hours. At different periods of time usually one hour, one of the Erlenmeyer flasks was removed and a loop of its contents used in the preparation of a slide. The staining of the slide was accomplished by the standard malachite green and safranin method. The spores appear green and the vegetative cells pink under the microscope. It was observed that after approximately 24 hours the majority of the vegetative cells were converted into spores or, at least, had endospores, but the incubation in the Dubnoff was continued for 24 hours more. Finally the five cultures were taken from the Dubnoff and kept in the refrigerator until ready for use.

All five cultures were combined in an autoclaved centrifuge bottle with its corresponding cotton plug, and then heated in a water bath at 80°C. for 25 minutes. This heat-treatment served the double purpose of

killing any vegetative cells which might have been still alive, and to activate the spores. The procedure then followed is outlined below.



After the final resuspension in saline, a 5 ml. sample of the spore suspension was transferred into a Klett tube, and its turbidity was measured using saline solution as the blank. The turbidity was 250 K.U.

Determination of the Basic Amino Acids for Spore Germination and Outgrowth: Different dilutions of the spore suspension prepared above were tested as inoculum with the five basic amino acid medium for vegetative cells, but no growth was observed even after 72 hours of incubation at 37°C. To determine if the lack of growth was because the spores were killed during their preparation, some nutrient broth was prepared and inoculated with the spore suspension (250 K.U.). At the end of 18 hours, considerable growth was observed on this medium. It was obvious from these results that something should be added to the five basic amino acid medium for vegetative cells in order to get the germination and outgrowth of the spores. After a short search in the literature, it was decided to test the effect of L-tyrosine, L-alanine, DL-alanine, adenosine, and biotin.

It also seemed desirable to test the effect of added manganese ion. For this purpose, Mn^{++}, in the form of $MnSO_4.H_2O$ was added to the non-amino acid medium in a concentration of 1.2 gm./ liter of non-amino acid medium. The five basic amino acid medium is thus modified for this one experiment to contain manganese ion at a concentration of 2.4 mg. $MnSO_4.H_2O$ per culture tube. Biotin was added by means of a loop, previously flamed in a Bunsen burner, to the autoclaved culture media. The amino acids and adenosine were each added at a concentration of 100 γ/tube. Some difficulty was encountered with precipitation of the Mn^{++} salts which interfered with the readings. The results are given in Table XI for 18 hours growth.

Based on the results below, a new experiment was devised to determine if the concentration of Mn^{++} might be decreased without decreasing the growth, thus eliminating the precipitation errors which introduce some uncertainty in the turbidity readings. A $MnSO_4.H_2O$ solution was prepared at a concentration of 1.2 gm./ liter. As usual, 2 ml. of the non-amino acid medium was used per culture tube. As it was desired to add the Mn^{++} to the amino acid medium without the other non-amino acid components in order to avoid the precipitation, 2 ml. (100%), 1 ml. (50%), 0.5 ml. (25%), and 0.2 ml. (10%) were added in duplicate to 4 pairs of culture tubes containing the amino acid medium. Then their volume was made up to 6 ml. and they were autoclaved at 15 pounds for 10 minutes. Then 2 ml. of the previously autoclaved non-amino acid medium was added to each of the culture tubes. A heavy salts precipitation was observed in tubes containing 100%, and 50% Mn^{++} and a slight precipitation occurred when the concentration was 25%. No precipitate was observed in the 10% Mn^{++} tubes.

Table XI

SPORE GERMINATION AND OUTGROWTH IN Mn^{++} CONTAINING MEDIA OF DIFFERENT AMINO ACID COMPOSITION

Amino Acid Medium Composition	K.U.
5 basic amino acids* + azide	0
5 basic amino acids + azide	0
5 basic amino acids	66
5 basic amino acids	63
5 basic amino acids + L-Tyrosine	71
5 basic amino acids + L-Tyrosine	71
5 basic amino acids + Adenosine	56
5 basic amino acids + Adenosine	57
5 basic amino acids + L-Alanine	66
5 basic amino acids + L-Alanine	69
5 basic amino acids + L-Alanine + L-Tyrosine	76
5 basic amino acids + L-Alanine + L-Tyrosine	76
5 basic amino acids + L-Alanine + Adenosine	60
5 basic amino acids + L-Alanine + Adenosine	60
5 basic amino acids + L-Tyrosine + Adenosine	78
5 basic amino acids + L-Tyrosine + Adenosine	75
5 basic amino acids + DL-Alanine	71
5 basic amino acids + AL-Alanine	66
5 basic amino acids + Biotin	60
5 basic amino acids + Biotin	58
5 basic amino acids + L-Alanine + L-Tyrosine + Adenosine	77
5 basic amino acids + L-Alanine + L-Tyrosine + Adenosine	72
5 basic amino acids + DL-Alanine + L-Tyrosine + Adenosine	73
5 basic amino acids + DL-Alanine + L-Tyrosine + Adenosine	78

* Modified to contain 2.4 mg. $MnSO_4 \cdot H_2O$ per culture tube

At the same time the effect of higher concentrations of L-alanine, L-tyrosine, and adenosine as germinating agents in the presence and absence of Mn^{++} was studied. All these compounds were used at a concentration of 800 γ/tube instead of the 100 γ/tube used previously. It was found that L-alanine and DL-alanine exhibit a strong stimulatory activity as germinating agents, as is shown in Table XII. The inoculation was carried out with one drop/tube of the spore suspension (250 K.U.) using a 3 ml. sterile pipette.

Table XII

SPORE GERMINATION AND OUTGROWTH IN DIFFERENT MEDIA

Composition of the Medium	K.U.
5 basic amino acids + azide	0
5 basic amino acids + azide	0
5 basic amino acids + 100% Mn^{++}	128
5 basic amino acids + 100% Mn^{++}	116
5 basic amino acids + 50% Mn^{++}	92
5 basic amino acids + 50% Mn^{++}	92
5 basic amino acids + 25% Mn^{++}	69
5 basic amino acids + 25% Mn^{++}	68
5 basic amino acids + 10% Mn^{++}	55
5 basic amino acids + 10% Mn^{++}	58
5 basic amino acids + L-Alanine + 10% Mn^{++}	112
5 basic amino acids + L-Alanine + 10% Mn^{++}	112
5 basic amino acids + L-Tyrosine	3
5 basic amino acids + L-Tyrosine	3
5 basic amino acids + DL-Alanine	74
5 basic amino acids + DL-Alanine	69
5 basic amino acids + Adenosine	6
5 basic amino acids + Adenosine	6
5 basic amino acids + L-Alanine	69
5 basic amino acids + L-Alanine	73
5 basic amino acids, no Mn^{++}	0

At this point it was decided to measure the growth of spores of B. cereus (569) in a medium containing the 5 basic amino acids required for the vegetative cells plus DL-alanine (6 basic amino acids medium for spores). The concentration of each of these amino acids in the final culture medium was 800 γ/ml. Mn^{++} was not added, neither in the non-amino acid medium, nor in the amino acids medium.

Another medium containing the 5 basic amino acids plus L-cysteine was also tested. L-Cysteine was added in a concentration of 800 γ/tube, and it showed a very strong stimulation of the germination and further outgrowth of the spores. One drop/tube of the spore suspension was used as inoculum. The growth was stopped 18 hours after the inoculation by the addition of two drops of 5% Na azide. The results of these two experiments are shown in Table XIII.

Table XIII

SPORE GERMINATION AND OUTGROWTH IN AN ALANINE CONTAINING MEDIUM

Amino Acids Composition of the Medium	K.U.
5 basic amino acids + azide	0
5 basic amino acids + azide	0
5 basic amino acids + DL-Alanine	92
5 basic amino acids + DL-Alanine	92
5 basic amino acids + L-Cysteine	74
5 basic amino acids + L-Cysteine	74
5 basic amino acids	0
5 basic amino acids	0

Effect of the Concentration of the Inoculum on the Germination and Outgrowth of Spores

The purpose of this experiment was to determine the effect of using spore suspensions of different concentrations in the inoculation of the culture media. The six basic amino acid medium was used in each case, and the growth was measured after 18 hours.

Spore suspensions of B. cereus (569) which read 49, 99, and 150 K.U. were prepared by pipeting, with sterile pipettes, 0.7, 1.7, and 3.3 ml. of our stock spore suspension into three different sterile culture tubes with cotton plugs. The volume was made up to 5 ml. with autoclaved saline solution, and after suspending the spores in the tubes, the turbidity of the suspension was measured in a Klett Photoelectric Colorimeter at 540 mμ. The turbidity of 5 ml. of the stock suspension of the spores was also measured, and it was found to have decreased in turbidity since its preparation. Originally its turbidity was 250 K.U. and three weeks later it was 216 K.U. This may have been due to a partial germination of the spores kept in saline solution. Despite this, the morphological appearance of the spores under the microscope did not show any change.

The inoculation was carried out by means of sterile 3 ml. pipettes, because in this way the size of the drop used in the inoculation was uniform, and there would be enough inoculum for any larger run. One drop was used per culture tube and the readings were taken after 18 hours. It was found that the total growth varies with the concentration of the spore suspension used as inoculum. Some representative figures are given in Table XIV.

Table XIV

EFFECT OF THE CONCENTRATION OF THE INOCULUM ON THE GERMINATION AND OUTGROWTH OF SPORES

K.U. of Spore Suspension	K.U. of Growth
0	0
0	0
49	41
49	39
99	55
99	52
150	66
150	66
216	80
216	75

Effect of B Vitamins on the Spore Medium for B. cereus (569)

An experiment was run in which the effect of different vitamins of the B complex was tested. For this purpose, 1 ml. of a sterile solution containing a mixture of traces of thiamin, riboflavin, pyridoxal, pantothenic acid, folic acid, biotin, and vitamin B_{12} was added aseptically to two culture tubes containing the 6 basic amino acid medium which had been previously autoclaved. The inoculation was carried out using one drop/tube of the stock spore suspension, and the readings taken after 18 hours.

Table XV

EFFECT OF B VITAMINS ON THE SPORE MEDIUM FOR B. CEREUS (569)

Vitamin Content	K.U.
None + azide	0
None + azide	0
None	59
None	59
Vitamin Solution	27
Vitamin Solution	25

From the results given in Table XV, it appears that the presence of B vitamins inhibits the growth of B. cereus (569), possibly because the vitamins solution used might have been too concentrated.

Minimal Inorganic Medium for Vegetative Cells and Spores

The effect produced by changing the composition of our non-amino acid medium was measured in this experiment. As amino acid medium, the five basic and the six basic amino acid media for vegetative cells and for spores respectively were used. In order to avoid any possible error produced by a difference in the degree of caramelization during the autoclaving of the non-amino acid media, a respective blank of each one of the media tested was prepared.

Before vegetative cells were inoculated in the medium contained in culture tubes, the cells were first centrifuged down, resuspended and washed three times with saline solution in order to eliminate any possible

traces of the substances to be studied. The inoculation was carried out by means of a 5 ml. hypodermic syringe in the case of the vegetative cells, and a 3 ml. pipette when spores were to be inoculated. The readings were taken after 18 hours. The results are sumarized in Table XVI.

Table XVI

DIFFERENT NON-AMINO ACID MEDIA AND THEIR EFFECT ON THE GROWTH OF B. CEREUS (569)

Compound Left Out	Compound Added	Concn. of the Addendum	K.U.
For Vegetative Cells			
Na Citrate	-	-	107
Na Citrate	Versene	8 gm./liter	0
$MgSO_4 . 7H_2O$	-	-	0
$MgSO_4 . 7H_2O$	$MnSO_4 . H_2O$	1.2 gm./liter	2
0.16% $Fe(NH_4)_2(SO_4)_2$	0.16% $(NH_4)_2SO_4$	24 ml./liter	113
Normal growth when all the original components were present			113
For Spores			
Na Citrate	Versene	8 gm./liter	0
$MgSO_4 . 7H_2O$	-	-	0
$MgSO_4 . 7H_2O$	$MnSO_4 . H_2O$	1.2 gm./liter	0
0.16% $Fe(NH_4)_2(SO_4)_2$	0.16% $(NH_4)_2SO_4$	24 ml./liter	20
Normal growth when all the original components were present			57

Versene, a strong metal-chelating agent, apparently binds the metals so tightly in the solution that the bacteria cannot utilize them. The complexes formed with citrate are apparently not so tight as those formed by versene, since the growth is only slightly different from the normal

growth when citrate is absent from the medium. It looks as if Mn^{++} cannot replace Mg^{++} in the medium, neither for vegetative cells nor for spores of B. cereus (569), and that Mg^{++} is essential. Fe^{++} had no apparent effect on the growth of vegetative cells, but it has a definite effect on spore germination and further outgrowth.

Inhibitory Studies with B. cereus (569)

Inhibition of Vegetative Cells of B. cereus (569) by β-2-Thienylalanine: In all the experiments to be described where vegetative cells were used as inoculum, the amino acid medium was our five basic amino acid medium, and for the spores, the six basic amino acid medium was used. All the cultures were grown in capped culture tubes, previously washed with "Tide", tap water, and distilled water, and dried in an oven at 120°C.

0.4 ml. of the five basic amino acid solution, concentration 16 mg./ml., was pipetted into each of the culture tubes previously treated as described above. Then different increments of a β-2-thienylalanine (β-2-TA) solution of a concentration of 500 γ/ml. were added to each of the culture tubes as shown in Table XVII. The volume of each tube was made up to 6 ml. with distilled water; the tubes were capped and autoclaved at 15 pounds of pressure for ten minutes. 2 ml. of the non-amino acid medium prepared as described in Table VII was added aseptically by means of a delivery pipette. This non-amino acid medium was previously autoclaved at 15 pounds of pressure for five minutes. After 24 hours of incubation at 37°C., the culture tubes were inoculated from a culture broth (70 K.U.) of the same composition as our minimal medium, using five drops from a 5 ml. hypodermic syringe. Every culture tube was flamed before and after the inoculation. The growth after 18 hours was measured turbidimetrically

in a Klett Photoelectric Colorimeter at 540 mμ, using sterile medium as the blank. In order to stop the growth, two drops of a 5% Na azide solution was added to each of the culture tubes at the end of 18 hours. A plot was made of growth (K.U.) vs. γ/tube of the inhibitor and is depicted in Figure 4.

In order to determine more precisely what amount of the inhibitor (β-2-TA) would produce a 50% inhibition of the growth, a similar experiment to the one described below was run. The inhibitor concentration ranged from 0 to 15 γ/tube. It was found that about 5 γ/tube of β-2-TA produced a 50% inhibition of the growth of the vegetative cells of B. cereus (569). The data is summarized in Figure 5. This time a 10 γ/ml. solution of β-2-TA was used. This solution was prepared by pipetting 1.0 ml. of the 500 γ/ml. β-2-TA solution prepared in the experiment above into a 50 ml. volumetric flask and making up to volume with distilled water.

Reversal by DL-Phenylalanine of the Inhibition Produced by β-2-Thienylalanine: An experiment to determine the approximate amount of DL-phenylalanine required to completely reverse the inhibition produced by 5 γ/tube of β-2-TA was run. Increasing amounts of DL-phenylalanine were pipetted into various culture tubes containing the amino acid medium plus 5 γ of the inhibitor. For this purpose, a 500 γ/ml. solution of DL-phenylalanine was prepared by weighing out 25 mg. of DL-phenylalanine and dissolving it in a 50 ml. volumetric flask. The volume was made up to 50 ml. with distilled water. An aliquot of 1 ml. was then transferred into another 50 ml. flask and diluted to volume with distilled water. This last solution was the one used in the experiment. The non-amino acid medium was of the same concentration used in the experiments before, and

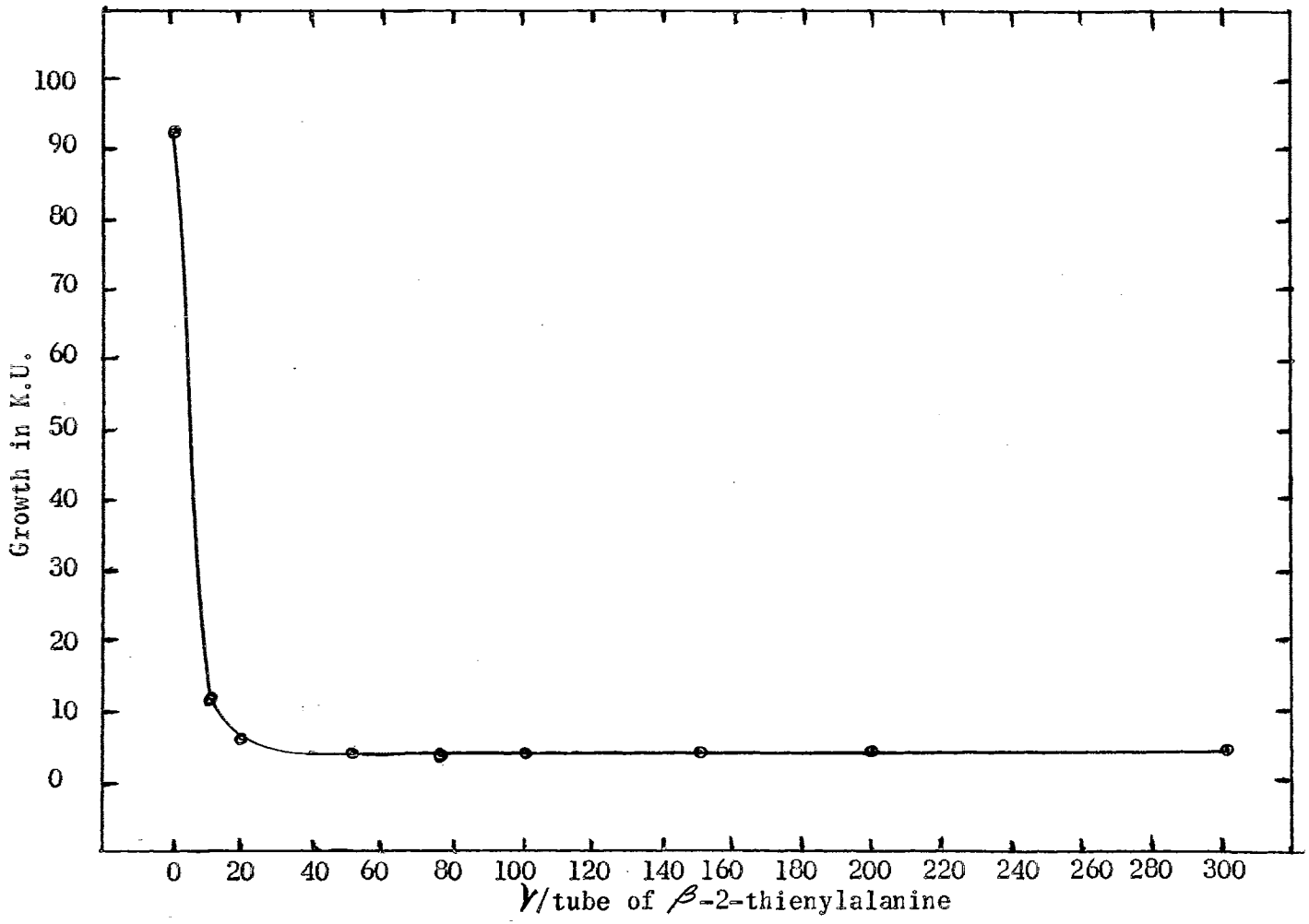


Figure 4. Ihnibition by β-2-thienylalanine of the growth of B. cereus (569)

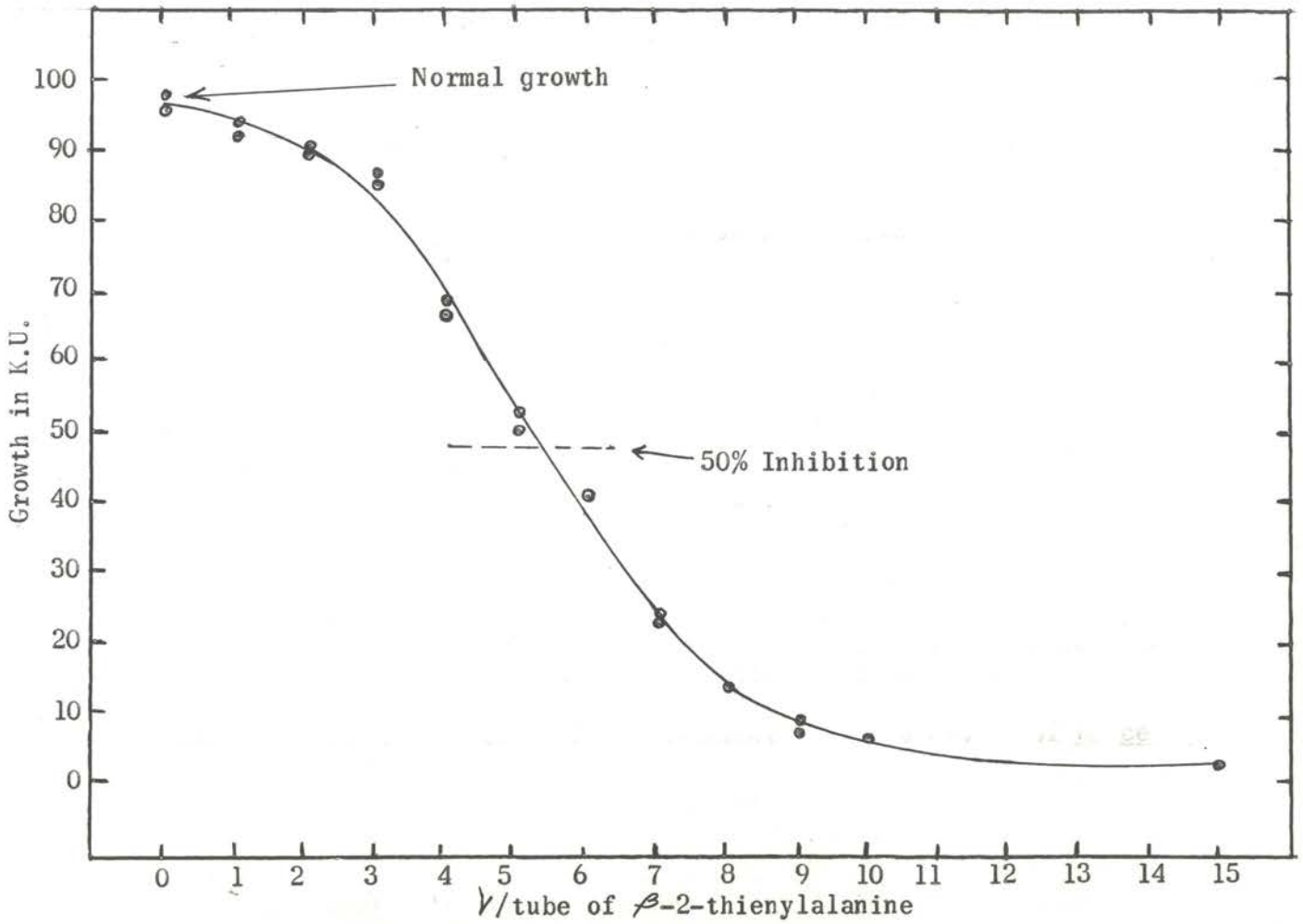


Figure 5. Inhibition by β-2-thienylalanine of the growth of B. cereus (569)

the same procedure was followed. The growth was measured turbidimetrically. A graph of K.U. vs. γ/tube of DL-phenylalanine is reproduced in Figure 6.

In order to determine the exact amount of DL-phenylalanine required to bring back the growth of B. cereus (569) to what we called normal growth, some other experiments were run, with DL-phenylalanine concentrations varying from 0 to 10 γ/tubes. Five γ/tubes of β-2-TA were used in every case. The procedure followed was the same as in the experiment above. The results are shown in Figure 7. It was found that the amount of reverser (DL-phenylalanine) required was 1.5 γ/tube when 5 γ of the inhibitor was added per culture tube. Since inhibition index is defined as the molar ratio of analog (β-2-TA) to metabolite (DL-phenylalanine) when one just nullifies the effect of the other, the inhibition index for this particular system was 3.10. The calculations are shown below.

$$\frac{\text{M of Antagonists}}{\text{M of Metabolites}} = \frac{5.0/171}{1.5/165} = 3.1$$

Reversal by L-Tyrosine of the Inhibition Produced by β-2-Thienylalanine: An experiment in which L-tyrosine was used as the reverser was performed. The solution of tyrosine used was of a concentration of 10 γ/ml. The general procedure followed was the same used for the reversal by DL-phenylalanine, but a complete reversal was not obtained in this case, probably because the main effect of the analog was to block the utilization of phenylalanine and not that of tyrosine. The results are depicted in Figure 8.

Inhibition by β-2-Thienylalanine of the Germination and Outgrowth of Spores: An inhibition experiment with β-2-TA was run to determine if the amount of inhibitor which produces a 50% inhibition of growth would be the same for both vegetative cells and spores. The six basic

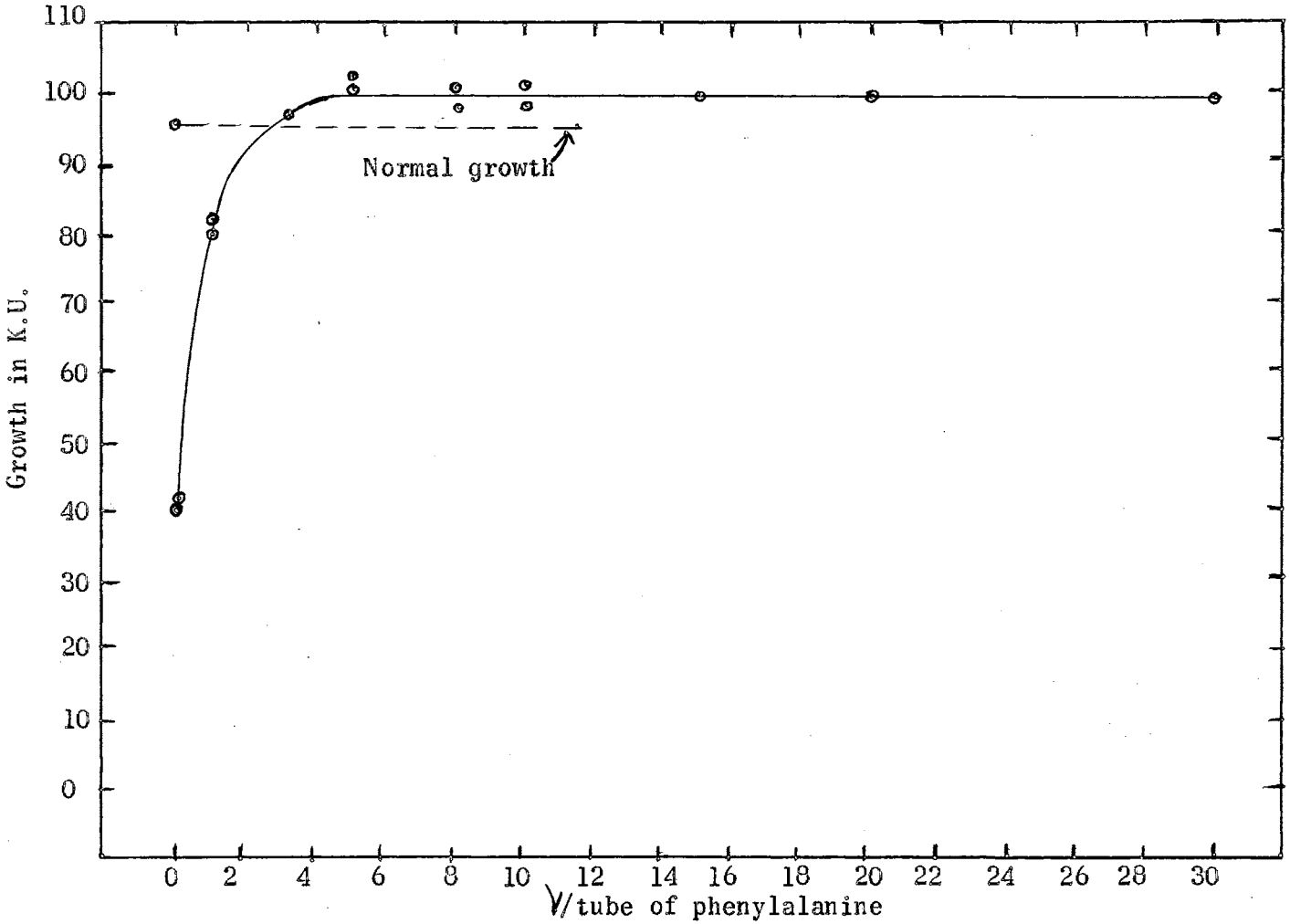


Figure 6. Phenylalanine reversal of the inhibition produced by 5 γ/tube of β-2-thienylalanine

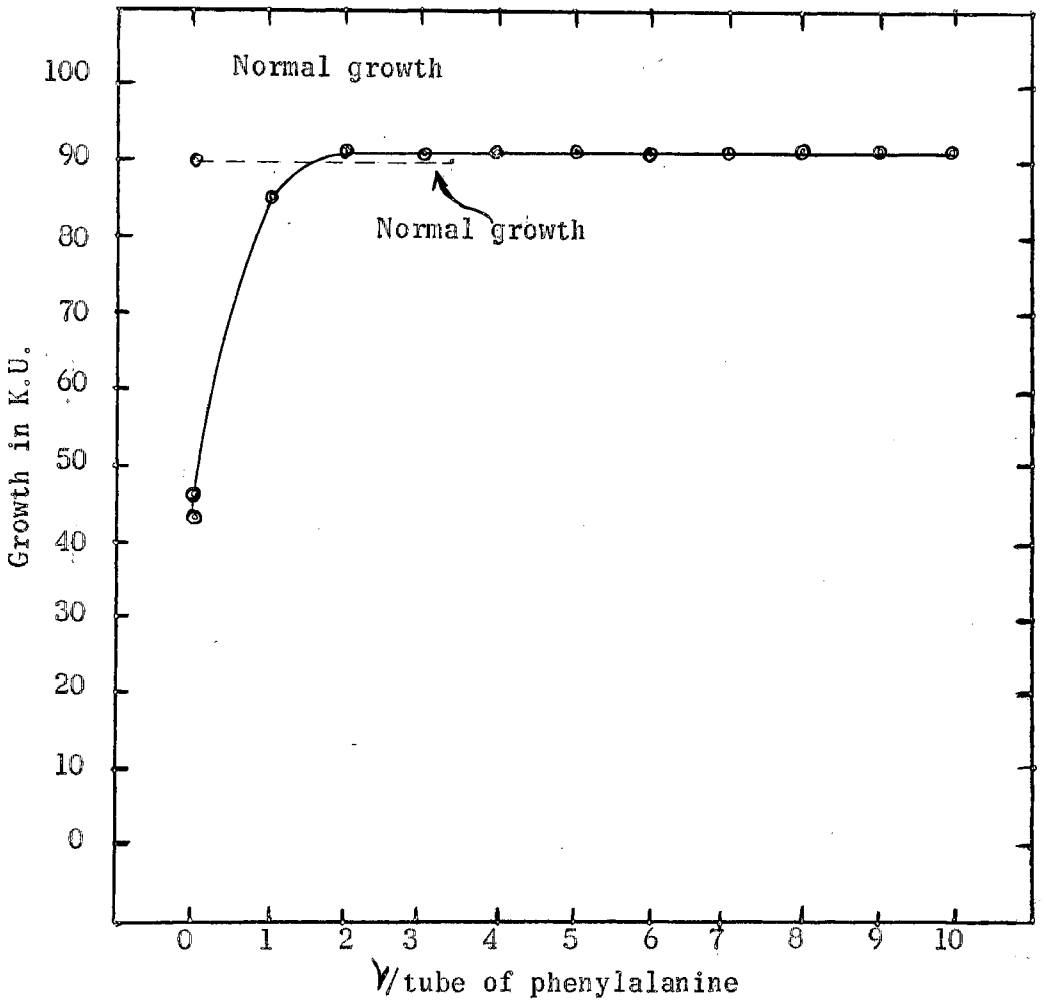


Figure 7. Phenylalanine reversal of the inhibition produced by 5 γ/tube of β-2-thienylalanine

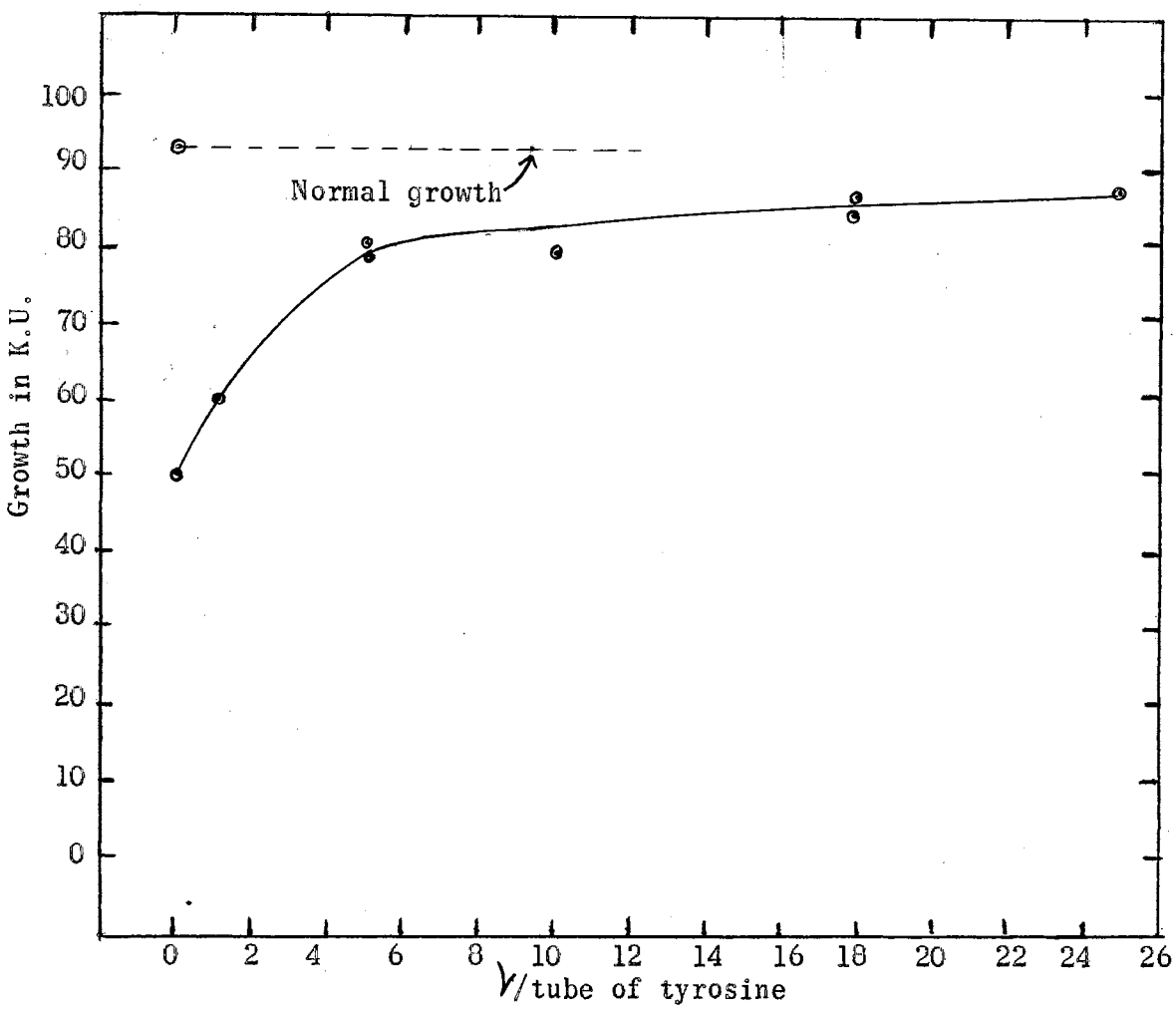


Figure 8. Tyrosine reversal of the inhibition produced by 5 γ/tube of β-2-thienylalanine

amino acid medium for spores was used, and the same technique used in previous experiments described in this work was followed. The results are given in Figure 9. A 10 γ/ml. solution of β-2-TA was the source of β-2-TA for this experiment. It was found that smaller amounts of the inhibitor are required to produce a 50% inhibition in B. cereus (569) growth when the medium is inoculated with spores.

Inhibition by Tryptazan of Vegetative Cells and Spores: Experiments of the same kind and following the same general procedure as those with β-2-TA were performed using tryptazan, a tryptophan analog, as the inhibitor. Typical results are shown in Figure 10. It was found that the amount of tryptazan which produces a 50% inhibition of the growth is 1.2 γ/tube.

Using spores and the six basic amino acid medium, an experiment was run following the same procedure used with β-2-TA. The findings showed that the amount of tryptazan which produces a 50% inhibition of the growth of B. cereus (569) was smaller for the spores than for vegetative cells. The data are depicted in Figure 11.

Reversal of Tryptazan Inhibition of Vegetative Cells with DL-Tryptophan: Following the same procedure used in the case of β-2-TA, but using DL-tryptophan as the reverser, it was found that the amount of the reverser required to bring back the growth to a normal level, when 1.2 γ/tube of the analog are present, is 0.5 γ/tube. Some of the results are presented in Figure 12. The inhibition index for this particular system, and as calculated from these results, was found to be 2.39.

Reversal of the Inhibition Produced by Different Levels of β-2-Thienylalanine and Tryptazan with Phenylalanine and Tryptophan Respectively: If the inhibition produced by different levels of β-2-thienylalanine

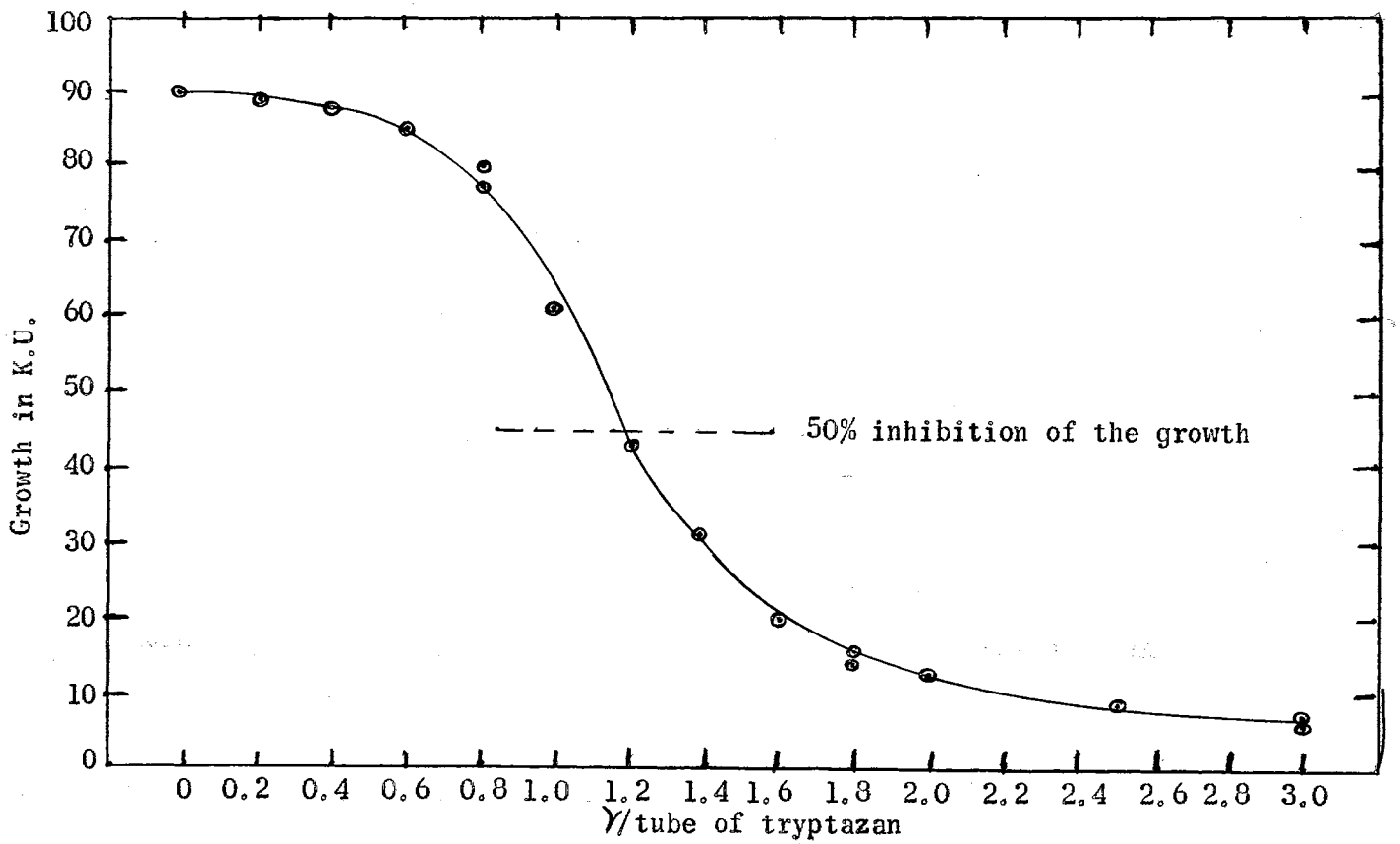


Figure 9. Inhibition by tryptazan of the growth of B. cereus (569)

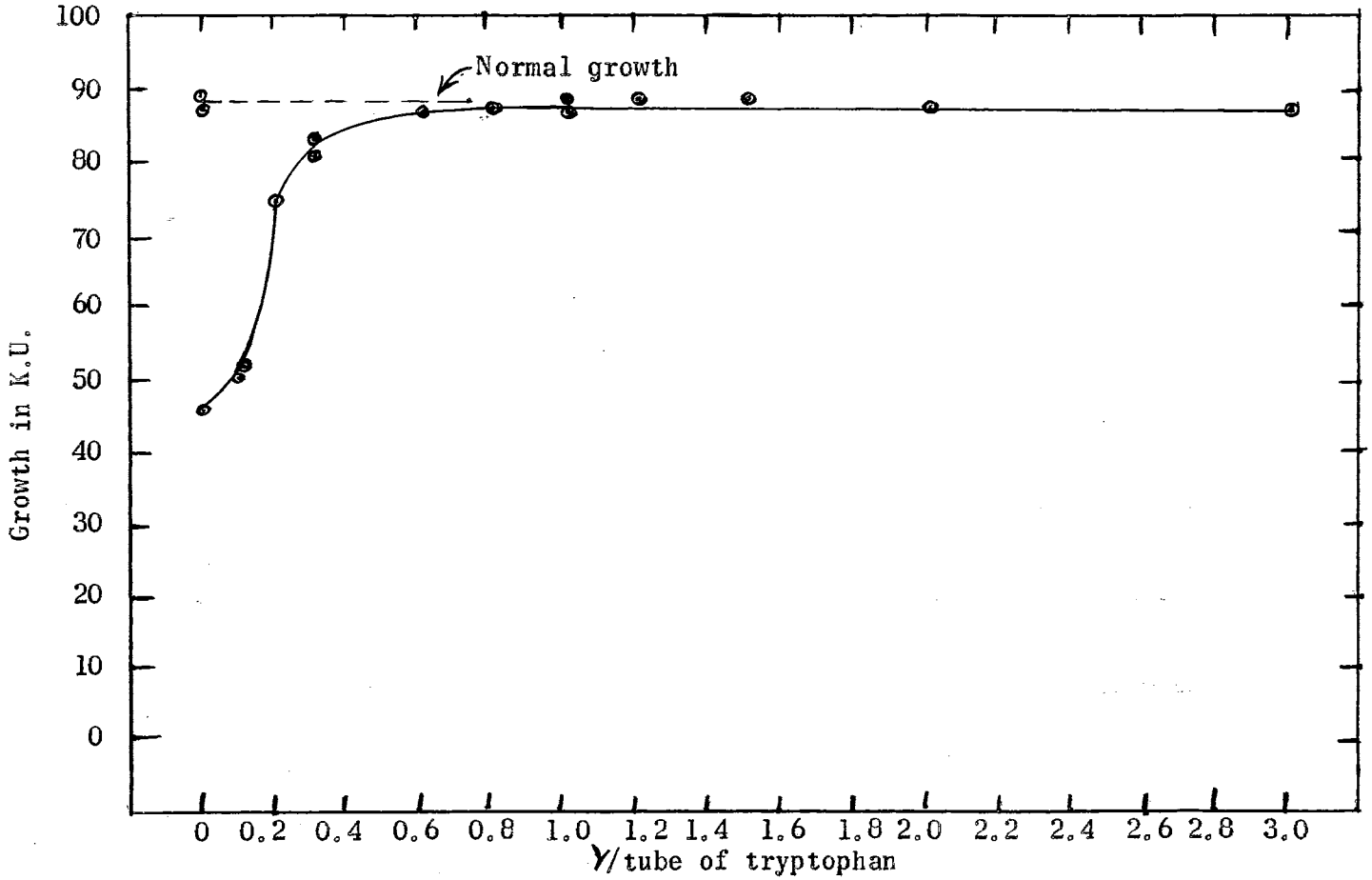


Figure 10. Tryptophan reversal of the inhibition produced by 1.2 γ/tube of tryptazan

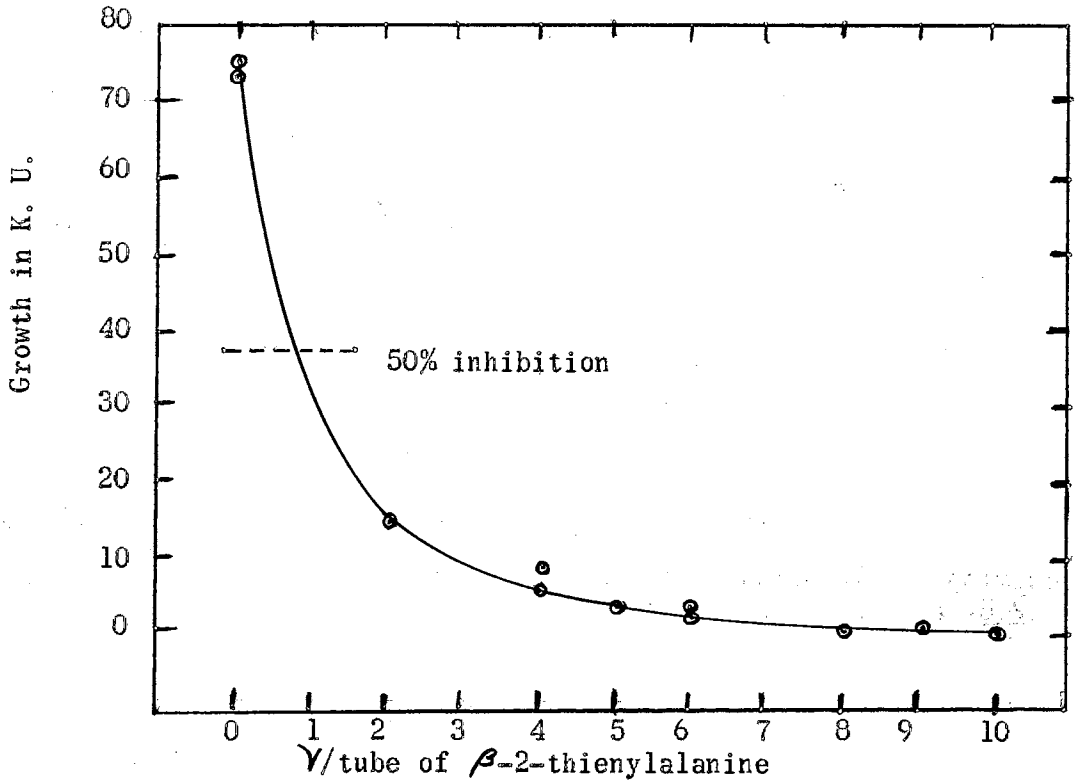


Figure 11. Inhibition by β-2-thienylalanine of the germination and outgrowth of spores of B. cereus (569)

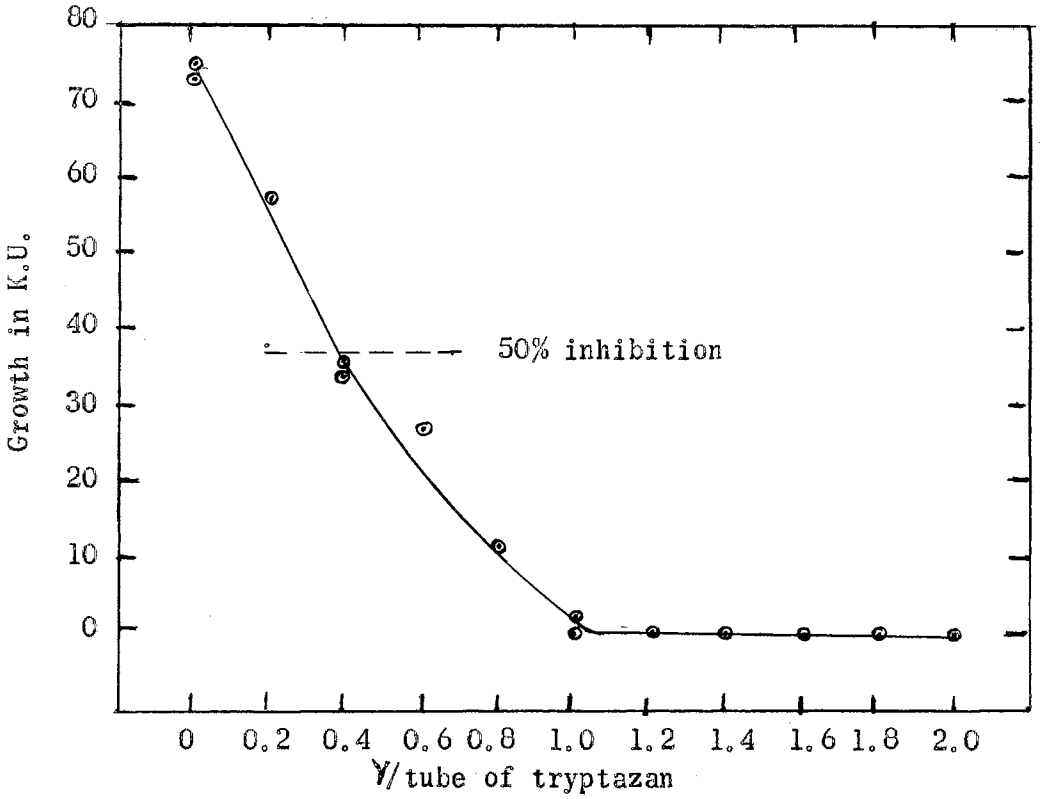


Figure 12. Inhibition by tryptazan of the germination and outgrowth of spores of B. cereus (569)

is of the competitive type, it was expected that the ratio of analog to metabolite would be constant. In order to determine whether this condition is satisfied, β-2-TA (or tryptazan) was added at three different levels (10, 15, and 20 γ) to the culture tubes containing the five basic amino acid medium for vegetative cells. Then DL-phenylalanine (or DL-tryptophan) was added in increasing concentrations as shown in Figures 13 and 14; the volume was made up to 6 ml. and autoclaved. After the addition of 2 ml. of the sterile non-amino acid medium, the culture tubes were inoculated from a broth with vegetative cells of B. cereus (569), and incubated for 18 hours. Finally the growth was stopped by the addition of two drops of a 5% Na azide solution to each culture tube, and the growth measured turbidimetrically in a Klett Photoelectric Colorimeter at 540 mμ.

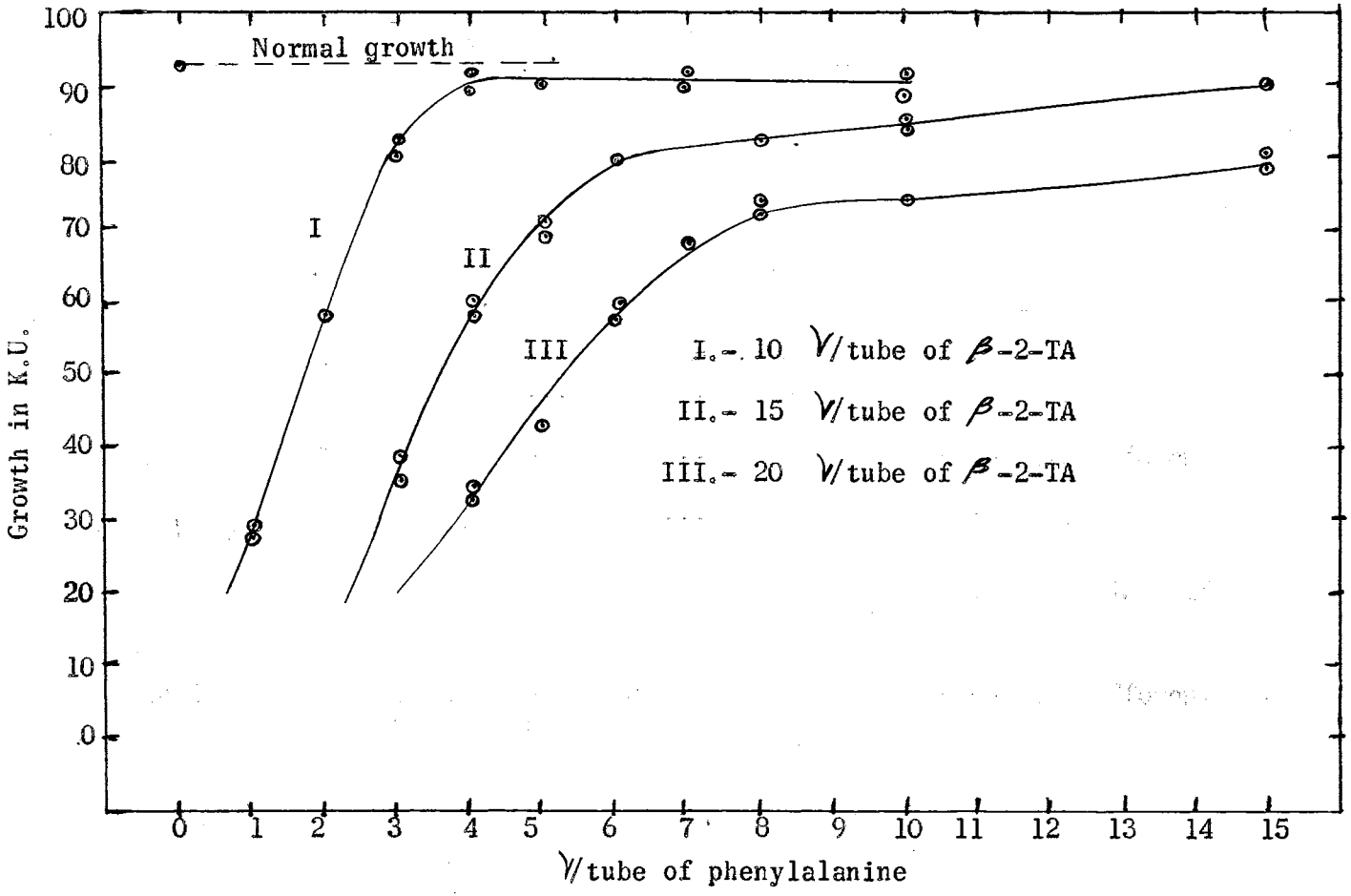


Figure 13. Phenylalanine reversal of the inhibition produced by different levels of β-2-thienylalanine

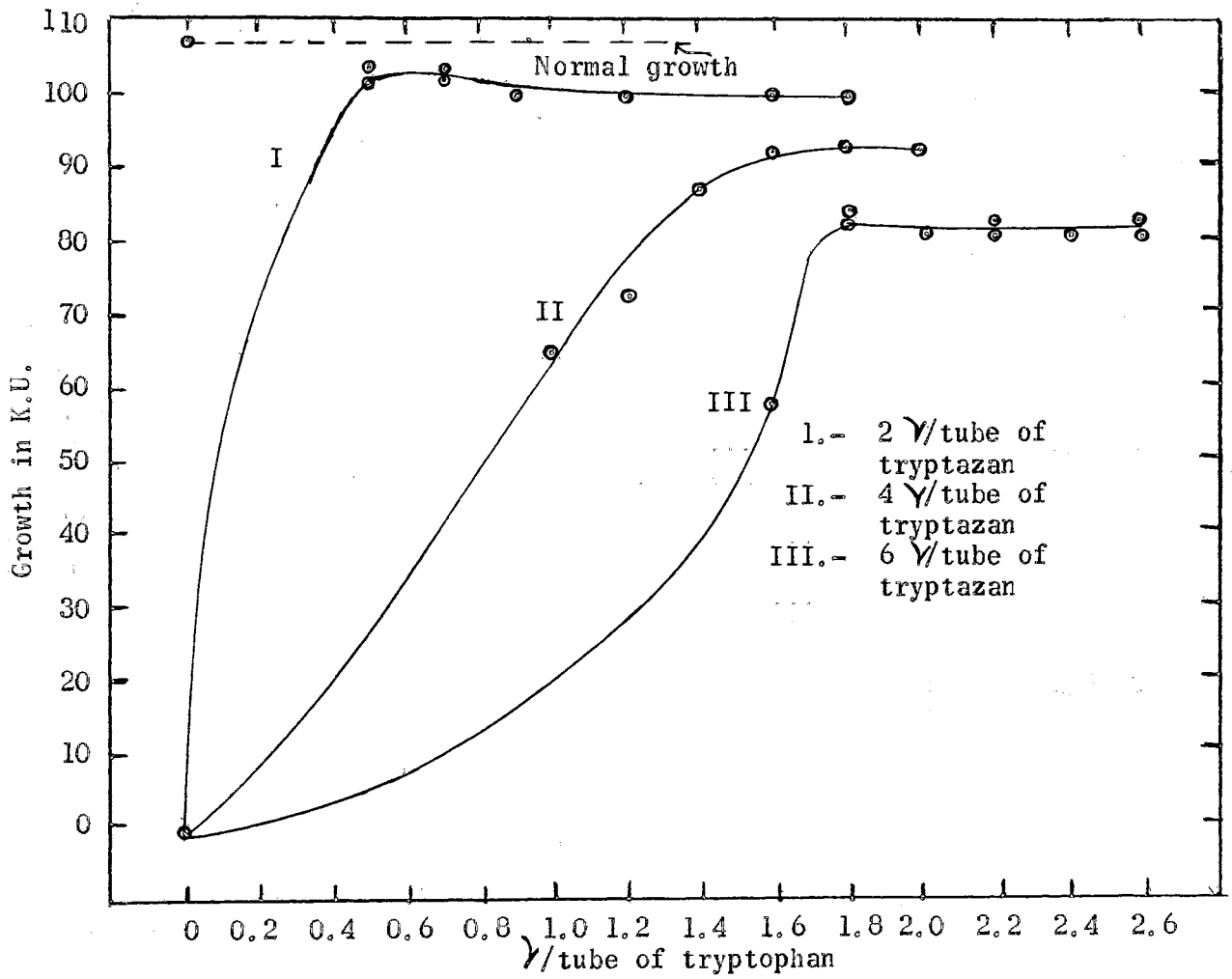


Figure 14. Tryptophan reversal of the inhibition produced by different levels of tryptazan

GENERAL DISCUSSION

There were two reasons for determining the correlation between the cellular dry weight of a culture and the turbidity produced by it. The results of this experiment indicate that there is a linear relationship between dry weight and turbidity at 540 mμ, at least up to 150 K.U. This correlation justifies the use of turbidity readings as a measure of growth. Also the data show that our selection of the range 80-100 K.U. as a reasonable amount of growth corresponds to a cell-dry-weight of 19 to 24 mg. per 100 ml. One of the points on our curve also checks closely with data of Collard and Knox (19). They used an inoculum with an optical density of 0.27, which corresponded to a cell-dry-weight of 27 mg./100 ml. In Figure 1 of this thesis, the same dry weight gives a turbidity of 110 K.U. which corresponds to an optical density of 0.22. Our slightly lower value could be due to a small difference in the length of the light path of the cuvettes used.

The fact that B. cereus (569) cannot use amino acids, to any large extent, as an energy source was very well established by the fact that in the absence of glucose and citrate the growth given in an 18 amino acid medium was very poor in comparison with that given when both glucose and citrate were present. This means that B. cereus (569) uses the glucose as its principal source of energy, and that the main use it makes of the amino acids is in the building of proteins.

During the course of the determination of the minimal medium for

vegetative cells, many interesting interrelationships among the several amino acids were found. It is very interesting that when all the 18 amino acids were present, the growth (normal growth) was about 159 K.U., but when amino acids like L-lysine, DL-methionine, DL-phenylalanine, L-proline, L-cysteine, DL-serine, DL-aspartic acid, and to a lesser extend glycine and DL-alanine, were omitted, a stimulation of the growth was obtained. These effects might be explained on the basis that some amino acids inhibit the utilization of the others. For example, it was found throughout this work that L-arginine was not an essential amino acid for the growth of B. cereus (569). This means that L-arginine can be synthesized by this microorganism, but when L-lysine is added to the culture medium, it might interfere with the utilization or with the synthesis of L-arginine. A different case is the one shown by the inhibitory effect of DL-aspartic acid, because it might be inhibiting L-glutamic acid, which was found to be an essential amino acid for B. cereus (569) (20).

The greatest inhibitory activity was shown by L-proline and L-cysteine. The inhibition shown by L-proline might be caused by its conversion into glutamine via pyrrolidone-carboxylic acid. The glutamine formed in this way could then act as an inhibitor for glutamic acid which was shown to be an essential amino acid for B. cereus (569) growth.

In the case of the inhibitory effect of DL-alanine and L-cysteine, these two amino acids might be blocking the utilization of glycine as has been found to be the case for microorganisms like Leuconostoc mesenteroides (21).

When the number of amino acids was cut down to ten, then five, and finally to four, the interrelationships among the several amino acids were found to be less obvious. The selection of our "basic" or "essential"

amino acids was based on their effects on the bacterial growth. For example, those whose omission from the test medium produced a decrease in the growth were selected for inclusion in the next, simpler medium. In this way there was a time when one medium only had four amino acids. Of these four amino acids, DL-valine was shown to be absolutely essential at all the levels tested. This is in accordance with the findings of Williams and Harper (3) that different strains of B. cereus grew only when valine was present in their culture media. When the number of amino acids was reduced to only five, there was no growth not only when DL-valine was omitted, but also when L-glutamic acid or DL-isoleucine were omitted, proving that these two amino acids also were essential under these conditions. The fact that isoleucine was a basic amino acid for the growth of B. cereus (569) is not unexpected considering the finding of O'Brien and Campbell (9) that isoleucine, valine and methionine were required for the growth of vegetative cells of B. cereus var. terminalis. The similarity of his findings to ours led us to test the effect of DL-methionine on the growth, and the results, once again were in accordance with those of the authors above.

Glycine exhibited a strong stimulatory effect on the growth, probably because it supplies the two-carbon units necessary for the synthesis of other amino acids like serine, and of other compounds like porphyrins, isocitrate, creatine, creatinine, glutathione, purines, and sarcosine.

It is interesting, that, when all the 18 amino acids tested were present, the requirement for glycine was not so evident as when only four amino acids were used. A similar pattern was exhibited by L-leucine and DL-isoleucine. At the beginning it appeared that leucine was more essential than isoleucine for the bacterial growth, but when the number of

amino acids in the medium was reduced to five, it was observed that the requirement of the microorganism for isoleucine was absolute, since there was no growth at all when DL-isoleucine was omitted from the medium. On the other hand, at this level, the omission of L-leucine had little effect. This might mean that in the presence of a more complex amino acid medium, DL-isoleucine omission is not so serious as omission of L-leucine simply because some other amino acid can be converted by the bacteria into isoleucine, but not into leucine. The conversion of threonine into isoleucine via α-keto-butyric acid is well-established (22), and analyzing our results, it is found that as soon as threonine is left out of the medium, the essentiality of isoleucine is manifested. This might be considered as an evidence that the explanation given is the correct one.

The finding that Mn^{++} is a very good activating agent of spore germination and further outgrowth is in accordance with the results of Levinson and Hyat (12), and as later it was found that alanine alone also has a similar effect, it could be that Mn^{++} activates a proteolytic enzyme, as proposed by Levinson and Hyatt, one of whose products is alanine. Since DL- and L-alanine were equally effective, this could mean that the activity of alanine racemase in the spores of this particular strain of B. cereus is quite high.

L-tyrosine and adenosine were also found to stimulate the germination and further outgrowth of B. cereus (569) spores, as had been previously indicated by Schmidt (10), but their effect was not nearly so great as that of alanine when relatively high concentrations were used. At low concentrations, L-tyrosine showed about the same activity as that shown by L-alanine and DL-alanine. L-cysteine also caused an appreciable stimulation, possibly by losing its -SH group and being converted to alanine.

B vitamins showed, in general, an inhibitory effect, which may be due to a superoptimal concentration of the vitamins used in the medium. This B vitamin effect was the same for both vegetative cells and spores of B. cereus (569).

Glucose was utilized as a very good carbon source in the culture medium; in its absence the growth was drastically decreased. The citrate functions simply as a metal ion complex-forming compound which prevents the precipitation of the metal ions of the salts added to the medium. Versene could not be substituted for Na citrate, apparently because it complexes the metals so tightly that they cannot be utilized by the bacteria.

Since, when Mg^{++} was left out of the medium there was a complete absence of growth, it appears to be absolutely essential for both vegetative cells and spores, and cannot be replaced by Mn^{++} because any time such replacement was made, there was no growth. This effect, produced by a lack of Mg^{++} in the system, may be the result of an inactivation of the numerous enzymes for which this ion is a cofactor. On the other hand, it seems from the results, that Fe^{++} is not essential for vegetative cell growth, but is essential for spore germination and further outgrowth, because in the case when vegetative cells were grown in a medium without Fe^{++}, a growth equal to the normal growth was obtained. When spores were used as inoculum, the growth was greatly decreased if Fe^{++} was not present.

With regard to the inhibition of B. cereus (569) growth by β-2-TA, and tryptazan, since the corresponding matabolites, DL-phenylalanine and DL-tryptophan, reversed the action of the antagonists, the inhibition is of a competitive type. Presumably, these metabolite antagonists act by inhibiting in one way or another specific enzymes systems which are still

unknown. That more than one enzyme system is inhibited by each of the analogs tested, is indicated by the fact that in both cases the corresponding metabolite failed to reverse the inhibition to a normal growth when different levels of the inhibitor were used. In the case of β-2-TA this failure may mean that the analog was not only competing with DL-phenylalanine but also with L-tyrosine, and possibly with L-histidine for the surface of the enzymes involved, as has been found in some other studies (20). A similar explanation may be given in the case of tryptazan, where the analog might be blocking the synthesis of nicotinic acid from tryptophan by the bacterium (22). This is consistent with the finding that nicotinic acid was not required for either vegetative cell or spore growth.

The spores were found to be more sensitive to the inhibitory action of the analogs than were the vegetative cells, since smaller amounts were required for 50% inhibition of spores. For example, 5 γ/tube of β-2-TA produced a 50% inhibition of the growth of vegetative cells, but 0.7 γ/tube of this antagonist were required when spores were used by the inoculum. A similar pattern was shown by tryptazan. It should be pointed out, however, that the spore medium was not the same, since it contained DL-alanine in addition to five basic amino acids required for vegetative cell growth. In other words, a six basic amino acid medium was used for spores.

SUMMARY

A minimal, chemically-defined medium for the growth of vegetative cells of B. cereus (569), containing 800 γ/ml. of DL-isoleucine, glycine, DL-valine, L-glutamic acid, and DL-methionine, was devised. This medium also contains glucose, NH_4Cl, Na citrate, $MgSO_4.7H_2O$, KH_2PO_4, and traces of Fe^{++} in the form of $Fe(NH_4)_2(SO_4)_2$. It appears that Fe^{++} is not essential for a normal growth response but Mg^{++} is required.

During the course of the determination of the minimal medium for vegetative cells, several interrelationships among the different amino acids tested were observed, and possible explanatory mechanisms are suggested.

For spore germination and outgrowth, only DL-alanine needs to be added to the five basic amino acids above, in order to get a normal growth. L-cysteine seems to have a similar effect to the one shown by L- and DL-alanine, and Mn^{++} presents a strong stimulatory acitvity. DL-tyrosine and adenosine showed a certain degree of stimulation, but the B vitamins did not.

β-2-thienylalanine and tryptazan, which are amino acid analogs of phenylalanine and tryptophan respectively, were tested. It was found that the inhibition index for β-2-TA was 3.1, and for tryptazan it was 2.39. The effect of these inhibitors when spores, instead of vegetative cells, are used as inoculum, is different. A possible explanation

for such a difference is suggested. Both inhibitors appear to compete with more than one metabolite in the bacterial body because when different levels of the inhibitor were used, the corresponding reverser failed to bring the growth to a normal level.

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