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TITLE A Synthetic Medium For Microbiological Assay Of Riboflavin, Pantothenic Acid, Biotin, Nicotinic Acid, Pyridoxine And Folic Acid

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A SYNTHETIC MEDIUM FOR MICROBIOLOGICAL ASSAY OF RIBOFLAVIN, PANTOTHENIC ACID, BIOTIN, NICOTINIC ACID, PYRIDOXINE AND FOLIC ACID. *

By

Andrew M. Hyma

The variables of casein hydrolysate in microbiological assay media were removed by the use of pure amino acids for the growth requirement of L. arabinosus and L. casei. Detailed studies were made on the minimum number and quantities of amino acids essential for growth of these organisms. In using L. arabinosus it was found that the following nine amino acids; tryptophane, cystine, glutamic acid, arginine, tyrosine, leucine, iso leucine, threonine and valine produced a good growth. The practical use of this medium for the assay of biotin, nicotinic acid and pantothenic acid was demonstrated with L. arabinosus as test organism. L. casei was more fastidious, and reduired in addition to the above amino acids factors present in yeast as biotin and folic acid. On addition of these factors L. casei gave consistent results on the assay of six factors of B complex vitamins as riboflavin, pantothenic acid, pyridoxine, nicotinic acid, biotin and folic acid. A mixture of all the amino acids, as occuring in casein hydrolysate, is recommended in order to balance oxidation and reduction reactions between pairs of amino acids because an absolute distinction cannot be maintained between H donating and H accepting amino acids. These amino acids in the quantities shown plus the purine and pyrimidine bases are thoroughly mixed in a desired amount and may be stored in amber bottles without apparent depreciation. The amount necessary for media preparation may be weighed from this mixture when and as required. In the use of this mixture the variables present in casein hydrolysate and yeast supplement are removed and consistent results are obtained. Studies were made on the effect of added carbohydrates, such as mannose, galactose, and inositol, as well as nucleic acid and para amino benzoic acid. The anti vitamin activity of methione also was investigated. The medium is of known chemical composition and results are more reproduciable and the procedure greatly simplified.

A SYNTHETIC MEDIUM FOR MICROBIOLOGICAL ASSAY OF RIBOFLAVIN, PANTOTHENIC ACID, BIOTIN, NICOTINIC ACID, PYRIDOXINE AND FOLIC ACID.*

By

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Introduction

Microbiological assays for the various factors of the B complex vitamins have become well established. These methods are based on the essential nature of the B complex factors as riboflavin, pantothenic acid, biotin, nicotinic acid, pyridoxine and folic acid for the initiation and maintenance of growth of certain types of bacteria. While many micro-organisms -- bacteria[1]; molds[2]; fungi[3]; are able to synthesize one or more of these factors, there are a few organisms such as the lactic acid bacteria, streptococci and others that are unable to grow unless an outside supply of one or more of the B complex factors are added. This has become the basis for the microbiological assay of the B complex vitamins. Lactic acid bacteria[4] and streptococci[5] are in current use for riboflavin assay and Proteus morgani[6, 7] lactic acid organisms[8, 9, 10, 11] and hemolytic streptococci[12] for pantothenic acid. Saccharomyces cerevisiae[13], Staphylococcus aureus[14], Clostridium butylicum[15] and Lactobacillus arabinosus[16] have been suggested and used for biotin assay. L. arabinosus[16] is also used for nicotinic acid determinations and Sacch. cerevisiae[17], Lactobacillus casei[18] for pyridoxine assay. Streptococcus lactis[19] and L. casei[20] are used in assay for folic acid.

From the above it becomes obvious that these microbiological assays are numerous with a wide variety of culture media and procedures. Although many of the above methods have become standard procedures a method which employs a simplified basal medium and a single test organism would be of great advantage. With this in mind the lactic acid bacteria have been investigated and L. casei and L. arabinosus have been employed. Very

satisfactory results have been attained with L. arabinosus for the assay of pantothenic acid, biotin and nicotinic acid. L. casei, however, gives the greatest promise as the desired test organism because its nutritional requirements include riboflavin[4], pantothenic acid[9], nicotinic acid[9], pyridoxine[21], biotin[20] and folic acid[20].

A study of media was made to devise a simplified medium that could be used for the assay of the various B complex vitamins. In practically all the procedures protein material in the form of casein, peptone and yeast is used in conjunction with certain amino acids. Hydrolyzed casein is a very prominent constituent of many of the media. Various hydrolysates of casein and gelatin have been used together with L. arabinosus and L. casei as test organisms.

A break down of the casein hydrolysate medium of Snell and Wright in their assay of nicotinic acid using L. arabinosus was made. This was done in order to investigate the constituents essential or responsible for growth. Their medium is of the following composition:

Acid hydrolyzed casein	0.5%
Cystine	0.01%
Tryptophane	0.01%
Glucose	1.0%
Sodium acetate	0.6%
Adenine	10 p.p.m.
Guanine	10 p.p.m.
Uracil	10 p.p.m.
Thiamine	0.1 p.p.m.
Vitamin B_6	0.1 p.p.m.

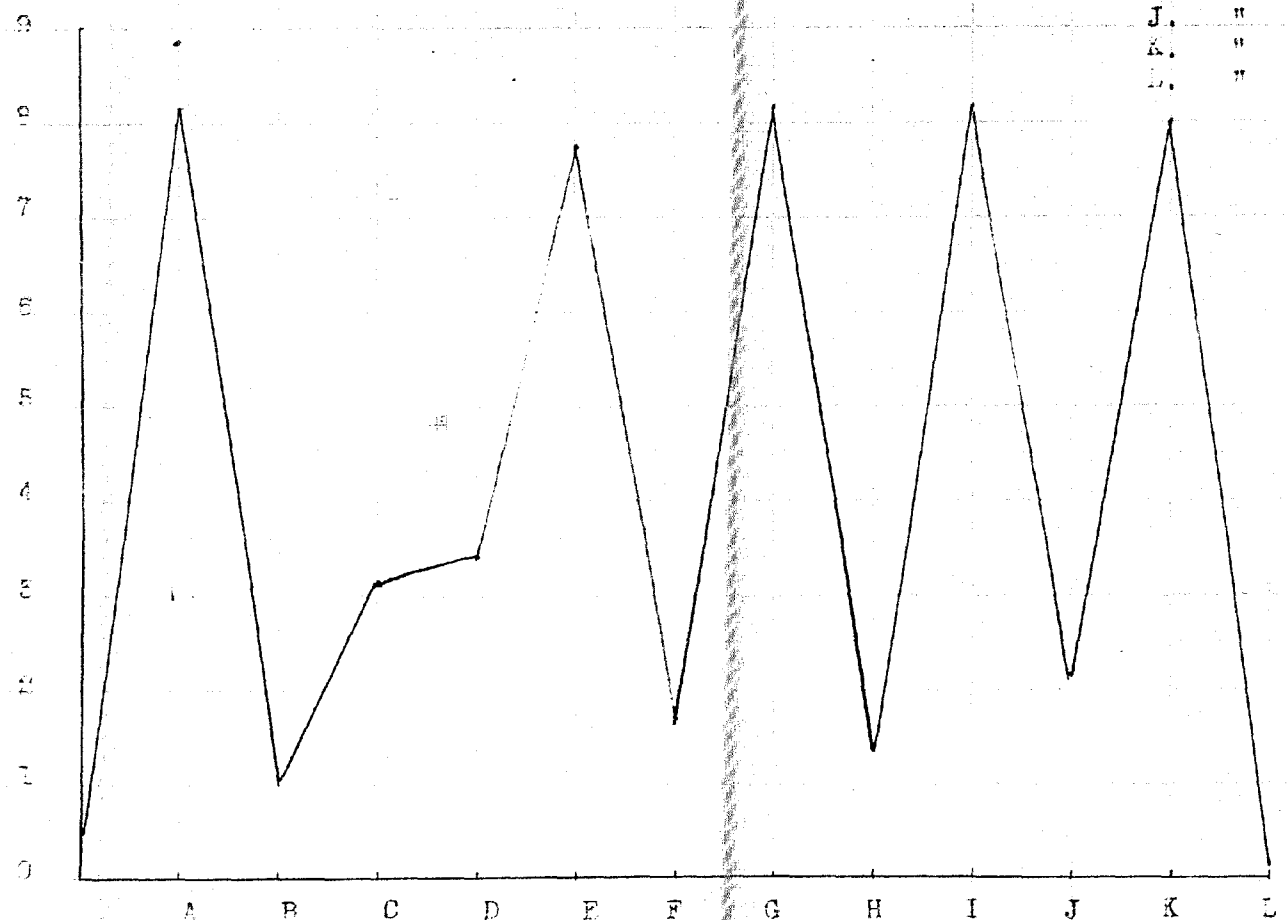
Riboflavin	--------	0.2 p.p.m.
Biotin concentrate	--------	0.4 p.p.billion of pure concentrate
Inorganic salts	--------	trace

Tryptophane was added by them because of its destruction on the acid hydrolysis of casein. Cystine was added because the amount naturally present in a casein hydrolysate is insufficient to meet the sulphur requirements of the test organism used. One constituent of the medium was omitted at each trial with the results as shown in the graph.

Thus it becomes evident that in addition to the B complex factors as pantothenic acid, biotin and nicotinic acid, this test organism requires the amino acids present in casein hydrolysate for growth. There is no growth on the omission of tryptophane. A small amount of growth response is noted on the omission of cystine. This indicates the presence of some cystine in the casein hydrolysate. The omission of casein hydrolysate results in no growth and shows the importance of the amino acids for growth response.

Under proper conditions, casein hydroly ate could be replaced by a mixture of purified amino acids, compounded to yield like results. Gladstone (23) in 1937 in a study of the nutritional requirements of Staph. aureus formulated a medium containing purified amino acids, with satisfactory results. The simularity of Gladstone's medium to the composition of casein hydrolysate is shown in Table 1.

The amount of casein hydrolysate and sodium hydroxide treated peptone generally recommended is 0.5 per cent[16, 20, 4, 8].



A. Shell & Height medium complete
B. Same minus Tryptophane
C. " " Cystine
D. " " Adenine guanine uracil
E. " " B_1
F. " " Ca Pentothenate
G. " " B_6
H. " " Biotin
I. " " B_{12}
J. " " Nicotinic Acid
K. " " $B_1B_2B_6$
L. " " Casein hydrolysate

TABLE I

Comparison of a one half of one per cent casein hydrolysate solution with Gladstone's medium per 10 cc.

Amino acids casein hydrolysate solution.	Mgs. per 10 cc.	Amino acids Gladstone's medium	Mgs. per 10 cc.
Glycine (0.4%) *	0.2	Glycine	0.1
Alanine (1.8%)	0.9	Alanine d l	0.4
Serine (0.5%)	0.25	Serine d l	0.2
Valine (7.9%)	3.95	Valine d l	1.6
Leucine) 9.7%		Leucine d l	1.0
Iso leucine)	4.85	Iso leucine d l	1.0
Phenylalanine (3.9%)	1.95	Phenylalanine d l	1.0
Tyrosine (6.5%)	3.25	Tyrosine l	0.7
Cystine (0.3%)	0.15	Cystine l	0.1
Tryptophane (2.2%)	1.1	Tryptophane l	0.3
Proline (8.0%)	4.0	Proline l	1.0
Hydroxy Proline (0.2%)	0.1	Hydroxy Proline l	0.1
Aspartic acid (4.1%)	2.05	Aspartic acid	0.5
Glutamic acid (21.8%)	10.9	Glutamic acid	2.5
Hydroxy glutamic (10.5%)	5.25	Hydroxy glutamic	
Histidine (2.6%)	1.3	Histidine l	0.4
Arginine (5.2%)	2.6	Arginine d	0.8
Lysine (7.6%)	3.8	Lysine di HL d l	2.0
Methionine (3.1%)	1.55	Methionine d l	0.8
Ammonia (1.6%)	0.8	Threonine d l	0.6

* Percentage of amino acid content of casein[22].

EXPERIMENTAL

The first investigations were concerned with the use of various hydrolysates. The procedure for the removal of vitamin factors was carried out according to standard practice[4, 8, 9, 10, 11]. Casein acid hydrolysate prepared from a vitamin free casein*, an enzymatic casein hydrolysate** and acid hydrolyzed gelatin were investigates in the preparation of standard basal media. The enzymatic casein hydrolysate gave a maximum acid production equivalent to 12.6 cc. of 0.1 N. sodium hydroxide. The acid hydrolyzed casein equivalent to 9.2 cc. and gelatin acid hydrolysate equivalent to 7.0 cc. Valine, tryptophane and hydroxy glutamic acid were added to the gelatin hydrolysate medium because they are not naturally present in gelatin. The amount of glutamic was increased to the amount present in casein hydrolysate as the amount present in gelatin is much less than in casein.

Varying results, however, were obtained depending upon the amount of Norite treatment for the removal of traces of vitamin content and the completeness of hydrolysis. Complete hydrolysis of casein is difficult. In the Norite treatment Tisiluis has reported the absorption of amino acids and peptides by activated charcoal and carbon[36]. Because of the incompleteness of hydrolysis, the variability in the composition of the original casein, and the loss of amino acids on vitamin removal, there is much uncertainty of the final amounts of each amino in the hydrolysate.

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* From: Research Laboratories, S.M.A. Corporation, Chagrin Falls, Ohio.

** From: Laboratories, Mead & Johnson, Evansville, Indiana.

It would appear, therefore, that there are too many variables in the final medium. Any change in quantity in each amino acid particularly if the amount is lower than the required minimun of the test organism would result in marked variations in assay results.

An assay test showed that the casein hydrolysate gave satisfactory results. However as stated, it is difficult to obtain hydrolysates of uniform composition. The next studies were logically examinations for the value of the amino acids present in casein hydrolysate, both as to the specific amino acid and quantities of each present. Accordingly media were prepared of pure amino acids based on the casein hydrolysate amino acid content. A determination was made of the essential amino acids necessary to produce and maintain growth by withholding, in turn, one of the amino acids present in casein hydrolysate. As far as possible the naturally occuring optically active forms of amino acids as found in casein were employed. Where it was necessary to use synthetic racemic forms the amounts were increased. A basal medium was first prepared according to Table II.

In previous experiments the need for tryptophane, cystine and casein hydrolysate have been demonstrated, as no growth occurs on the omission of any one of these constituents from the medium. The basal medium (Table II) with all the amino acids (19) present in the required amounts, as in casein hydrolysate, gave a maximum acid production equivalent to 9.8 cc. 0.1N NaOH. Results on the withholding of one of the amino acids in turn are shown on the accompanying graph.

There is practically no growth without tryptophane (2), cystine (3), glutamic acid (4) and valine (16) and very little growth without

TABLE II

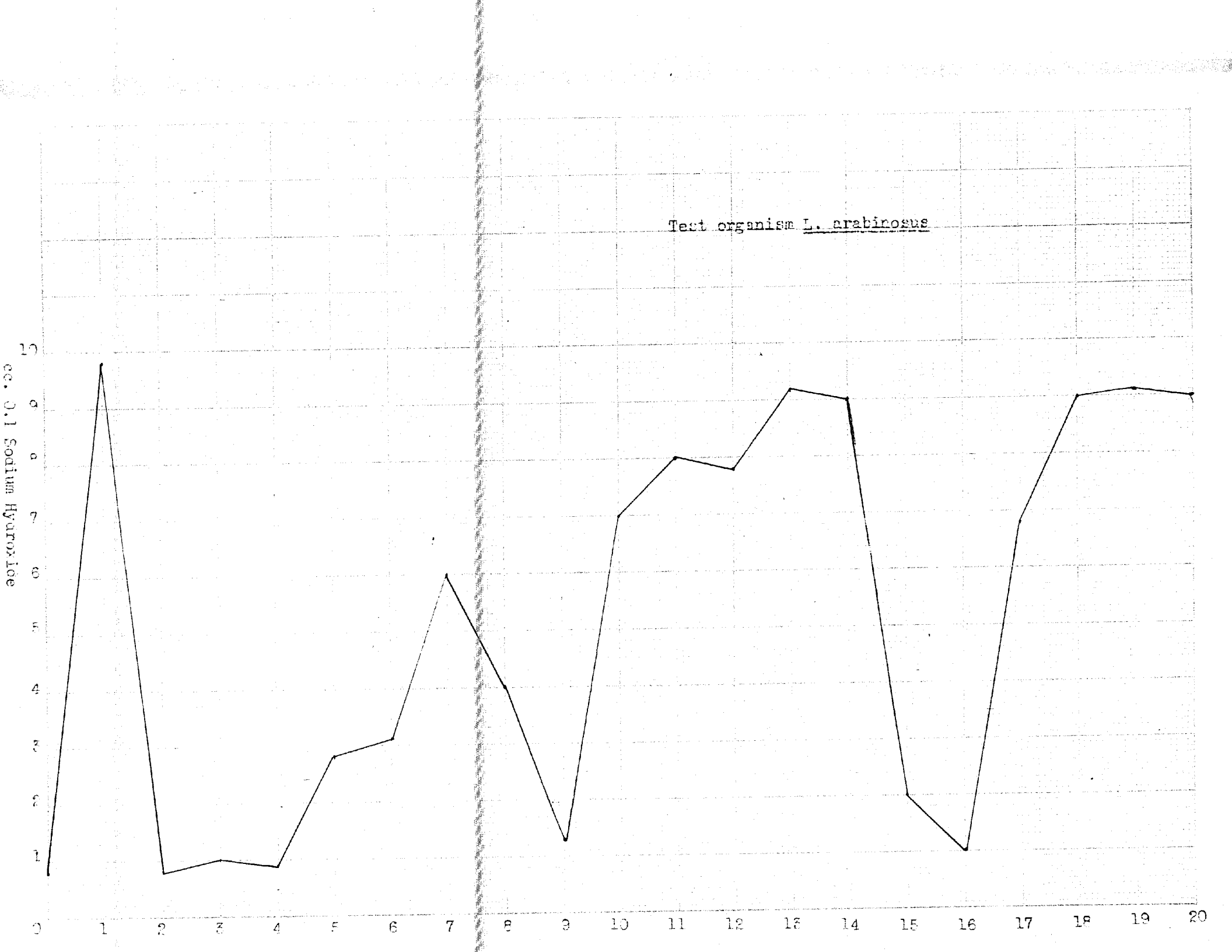
Lactic acid bacteria, L. casei and L. arabinosus were selected as test organisms. These organisms were selected because of their fastidious nutritional requirements.

l(-)	Tryptophane	0.100 gms.
l(-)	Cystine	0.100 gms.
	Glucose	10.0 gms.
	Sodium Acetate	6.0 gms.
	Soln. A* (Inorganic salts)	5.0 ml.
	Soln. B* (" ")	5.0 ml.
	Adenine	0.005 gms.
	Guanine	0.005 gms.
	Uracil	0.005 gms.
	Nicotinic acid	0.0001 gms.
	Thiamine hydrochloride	0.0001 gms.
	Calcium pantothenate	0.0001 gms.
	Pyridoxine	0.0001 gms.
	Riboflavin	0.0002 gms.
	Biotin (Free acid)	0.0002 gms.
	Yeast supplement**	10.000 cc.
	Water to	1000 cc.

(pH adjusted to 6.8)

*Soln. A		*Soln. B	
K_2HPO	5 mgs.	$MgSO_4.7H_2O$	10 gms.
KH_2PO_4	5 mgs.	NaCl	0.5 gms.
Water	50 cc.	$FeSO_4.7H_2O$	0.5 gms.
		$MnSO_4.4H_2O$	0.337
		Water	250 cc.

** The yeast supplement equivalent to 2.0 gms. of whole autolyzed yeast. Yeast was added for studies with L. casei.


Test organism L. arabinosus
cc. 0.1 Sodium Hydroxide
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Explanation of Graph

1. Basel medium Table II complete with all amino acids as in casein hydrolysate.
2. Basel medium Table II minus l - tryptophane
3. " " " " " l - cystine
4. " " " " " d - glutamic acid
5. " " " " " d - arginine
6. " " " " " l - tyrosine
7. " " " " " l - aspartic acid
8. " " " " " l - leucine
9. " " " " " l - isoleucine
10. " " " " " d l - phenyl alanine
11. " " " " " d l - alanine
12. " " " " " d - lysine
13. " " " " " l - serine
14. " " " " " l - histidine
15. " " " " " d l - threonine
16. " " " " " d l - valine
17. " " " " " l - methonine
18. " " " " " l - proline
19. " " " " " l - hydroxy proline
20. " " " " " glycine

arginine (5), tyrosine (6), aspartic acid (7), leucine (8), iso leucine (9) and threonine (15). The omission of phenyl alanine (10), alanine (11), lysine (12), methionine (17), cut down acid production to a great extent. Acid production was only lowered to the equivalent of less than 1 cc. of 0.1 N sodium hydroxide on the omission of serine (13), histidine (14), proline (18), hydroxy proline (19) and glycine (20). This would indicate that tryptophane, cystine, glutamic acid, isoleucine, leucine, valine, arginine, tyrosine, threonine are essential for growth of the lactic acid bacteria. The addition of aspartic acid, phenyl alanine and methonine increase acid production. Maximum production of acid is obtained when all the amino acids in the quantities present in casein hydrolysate are employed. This holds true only for L. arabinosus. Practically equivalent results occured when yeast supplement [32] was added to the medium in the case of L. casei.

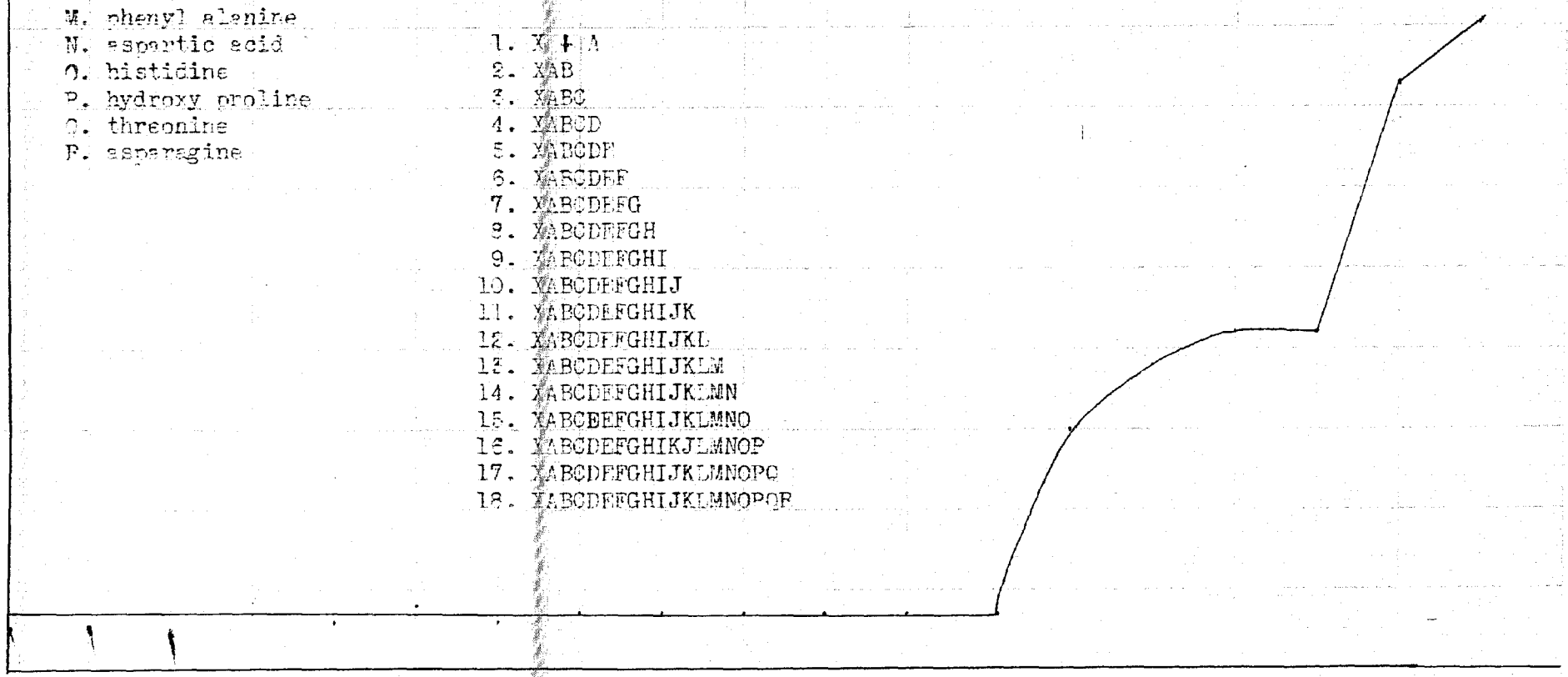
Further investigation of the nutrient requirements of L. casei and L. arabinosus for the various amino acids were carried out using individual amino acids in conjunction with the basal medium. Many combinations were tried in this experimentation. The basal medium employed is that shown in Table II above. To the basal medium was added one amino acid at a time to ascertain which amino acid would stimulate growth, and in such a manner that the resulting medium was augmented by each successive addition. To the basal medium was added (1) glutamic acid, the next test medium was (1) glutamic plus (2) lysine. Each addition was made in the order as shown on graph. While threonine is not reported as a natural constituent of casein hydrolysate [22] it is an aid in promoting greater growth. Gladstone [23] included it in his amino acid combination. Further it has been found that on a thorough

Series I

A..Glutamic acid
B. lysine
C. leucine
D. tyrosine
E. Arginine
F. glycine
G. proline
H. methienine
I. alanine
J. valine
K. serine
L. iso leucine
M. phenyl alenine
N. aspartic acid
O. histidine
P. hydroxy proline
Q. threonine
R. asparagine

1. X + A
2. XAB
3. XABC
4. XABCD
5. XABCDE
6. XABCDEF
7. XABCDEFG
8. XABCDEFGH
9. XABCDEFGHI
10. XABCDEFGHIJ
11. XABCDEFGHIJK
12. XABCDEFGHIJKL
13. XABCDEFGHIJKLM
14. XABCDEFGHIJKLMN
15. XABCDEFGHIJKLMNO
16. XABCDEFGHIKJLMNOP
17. XABCDEFGHIJKLMNOPQ
18. XABCDEFGHIJKLMNOPQR

tryptophane
tryptophane
cystine
Sodium acetate
glucose
adenine quanine
uracil
Sol'n. A.
Sol'n. B.
Sol'n. B_1B_6 Ca. Panto.
Sol'n. B_2
Sol'n. Biotin
Sol'n. Nicotinic acid



cc. 0.1 N. Sodium Hydroxide

investigation of the hydrolytic products of casein, a mixture of the 19 amino acids commonly occuring in proteins does not permit growth, or support uniform body weight in rats. On the addition of threonine to the above 19 amino acids, rats were able to support excellent growth. Omission of threonine resulted in a loss of body weight[36]. For these reasons and the fact that in previous experimentations, it had been found to be indispensable for growth of L. arabinosus, we included it in the investigation. Asparagine was also added because many investigators have used it in conjunction with acid hydrolyzed casein media.

No apparent growth was noted until the addition of phenylalanine (13) to the twelve preceding acids. Of the twelve acids first used no one amino acid or combination of the twelve produced growth. Phenylalanine in combination with the first twelve acids produced some growth which was brought up to a higher level by the addition of (14) aspartic acid, and still higher by the addition of (17) threonine. The addition of (15) histidine and (16) hydroxy-proline caused little increase in growth. Maximum growth was attained when a combination of all the amino acids through 17 were added. This would indicate that phenylalanine, aspartic acid, threonine and asparagine were growth promoting factors in conjunction with one or more of the following amino acids; glutamic acid, lysine, leucine, tyrosine, arginine, glycine, proline, methionine, alanine, valine, serine and isoleucine.

Another grouping of amino acids shows that proline, hydroxy-proline and alanine aid somewhat in growth production.

Four hundred and eight combinations of the various amino acids were run thereafter in triplicate with interesting results. It was

found that good growth could be attained by using a combination of the following seven amino acids in conjunction with the basal medium (which contains the amino acids, tryptophane and cystine). The seven amino acids are glutamic acid, arginine, tyrosine, leucine, isoleucine, threonine and valine.

Maximum growth and acid production were attained on the addition of methionine, aspartic acid, lysine, and phenylalanine. This combination of eleven amino acids plus the basal medium, (thirteen with tryptophane and cystine in the basal medium) produced excellent growth. The omission of any one of the 13 acids produced from practically no growth on the omission of tryptophane, cystine, valine, isoleucine, threonine, glutamic acid to from a 15 to a 60 per cent decrease on the omission of any of the others. Yeast extract had to be added to the medium in the case of L. casei to produce satisfactory results. L. arabinosus did not require the yeast supplement.

Wolley and Hutchings[24] have found that the simplest effective amino acid combination consisted of tryptophane, glutamic acid, isoleucine, lysine, arginine, tyrosine, cystine and methionine in the growth and acid production of Streptococcus zymogenes. Clifton[25] working with Clostridium botulinum[26] used a combination of alanine, proline, leucine, serine, glutamic acid, lysine, cystine and tryptophane. Wolley[26] found that seven amino acids, namely: glutamic acid, tryptophane, isoleucine, lysine, arginine, cystine and tyrosine are essential for growth of hemolytic streptococci. This would indicate that the amino acid requirements of the lactic acid bacteria are similar to the requirements of these micro-organisms. The simplest combination of

amino acids was found to consist of glutamic acid, arginine, tyrosine, leucine, isoleucine, threonine, valine, tryptophane and cystine. An interesting comparison with the finding of Rose[37] on essential amino acids for growth production may be made at this point. It was found that the following 10 amino acids; lysine, tryptophane, histidine, phenylalanine, leucine, isoleucine, threonine, methionine, valine and arginine are indispensable requirements for the growing animal. Although this combination is sufficient for growth of the lactic acid bacteria, the remaining amino acids in casein hydrolysate can be added without a great deal of difficulty and expense and the growth level raised. Furthermore the addition of all of the amino acids present in casein hydrolysate may aid to balance oxidation and reduction reactions between pairs of different amino acids as in the Stickland reaction[27].

Stickland[27] showed that washed suspensions of Clostridium sporogenes activate certain amino acids as hydrogen-donators and others as hydrogen-acceptors, so that coupled reactions take place between pairs of them resulting in their deamination. Woods[30] continued the study of this function. The results of these two workers show that the following amino acids act as H-donators: alanine, valine, leucine, phenylalanine, cysteine, serine, histidine, aspartic acid, and glutamic acid; and the following as H-acceptors: glycine, proline, hydroxy-proline and arginine. Stickland[27] also showed that reaction between glycine and alanine gives rise to two molecules of acetic acid, CO_2 and NH_3, and that l-proline is reduced by l-alanine in the presence of C. sporogenes to produce delta amino valeric acid plus pyruvic acid and NH_3.

Methionine[28] has been reported to be an anti-vitamin which inhibits growth due to its anti-biotin activity. A series of tests were run

omitting methionine from the mixture of glutamic acid, arginine, tyrosine, aspartic acid, leucine, isoleucine, phenyl alanine, lysine, threonine, tryptophane and cystine. The maximum growth attainable was less than 50 per cent on the omission of methionine. It has been found, however, that while methionine may retard growth at least for the first twenty-four hours, in the case of lactic acid bacteria, it aids greatly in promoting maximum growth. It did not appear to greatly hinder the growth of L. arabinosus which has been shown to require biotin for growth.

Para amino benzoic acid has been reported as a metabolite essential for bacterial growth[29]. No appreciable effect on growth was noted on the addition of para amino benzoic acid to the medium of amino acid combinations in the case of lactic acid bacteria.

Certain purified proteins have been shown to yield various amounts of carbohydrates probably polysaccharide composed of one molecule of glucosamine and two molecules of either galactose or mannose. It still remains to be proven whether or not carbohydrates are integral parts of the protein molecule[22].

Inositol, (Bios I), may also be of interest for bacterial growth stimulation. It is a substance necessary for the normal production of the yeast cell[31].

Experimentation using glucose 10 gms., mannose 5 gms. and galactose 5 gms. per 1000 cc. of basal amino acid medium was tried with indifferent results. A basal amino acid medium containing 10 gms. of glucose, plus 5 gms. of mannose, plus 5 gms. of galactose, plus 5 gms. of inositol was next tried; also media containing 10 gms. glucose and 5 gms.

of mannose; 10 gms. glucose plus 5 gms. of galactose; and 10 gms. glucose plus 5 gms. of inositol with no apparent growth increase. All this would indicate that added carbohydrates are not essential for the stimulation of growth and acid production of the lactic acid bacteria. These carbohydrates were added to ascertain their effect on growth and acid production in the media. Carbohydrates[38] have been reported to have a sparing action on the deamination of protein.

In some cases the presence of glucose has little or no effect upon actual deamination by washed suspensions grown in a tryptic digest of casein. In some instances it has been reported that carbohydrates have the opposite effect, that in place of checking the production of ammonia in protein digest, it increases NH_3 production and enables the bacteria to utilize more protein[38]. Deamination and decarboxylation are due to cell enzymes and not dependent upon carbohydrate content.

Xanthine was added to the mixture as a result of the findings of Snell & Mitchell[35], they report that the purine bases, adenine, guanine, xanthine and the pyrimidine base uracil are growth promoting substance for the lactic acid bacteria. Asparagine was also added. Penny and Strong[34] found that it was an aid in accelerating growth especially in the earlier part of the incubation period.

As a result of the amino acid requirement studies, it was possible to formulate for the assay medium, the amounts of each amino acid that gave maximum growth results. A stock mixture of the amino acids in their naturally occuring optical forms (where racemized forms are used the amount is doubled), plus adenine, guanine, uracil, xanthine, asparagine, was thoroughly mixed and prepared in proportions according to Table III.

TABLE III

Cystine l-	2.500 gms.
Adenine	0.025 gms.
Guanine	0.025 gms.
Uracil	0.025 gms.
Xanthine	0.025 gms.
Tryptophane l-	2.500 gms.
Asparagine	1.250 gms.
Glutamic acid d l	5.450 gms.
Arginine monohydrochloride l	1.300 gms.
Aspartic acid d l	2.050 gms.
Leucine l-	1.200 gms.
Isoleucine d l	2.400 gms.
Phenylalanine d l	1.950 gms.
Alanine d l	.900 gms.
Lysine monohydrochloride l	1.900 gms.
Serine d l	0.250 gms.
Histidine monohydrochloride l	0.650 gms.
Threonine d l	0.750 gms.
Valine d l	3.950 gms.
Methionine d l	1.550 gms.
Proline l -	2.000 gms.
Hydroxy proline l -	0.050 gms.
Glycine	0.100 gms.
Glutamic acid monohydrate d l	2.625 gms.
Tyrosine l-	1.625 gms.
Total	37.050 gms.

This combination of amino acids produced the best results on vitamin assays. The stock mixture has been kept securely capped in an amber bottle for several months without any apparent depreciation. This amount in the proportions named is sufficient to produce 2500 cc. of double strength amino acid basal medium. The basal medium is prepared as follows:

Amino acid mixture		7.410 gms.
Glucose		10.000 gms.
Sodium acetate		10.000 gms.
Salt Sol. A*		5.000 cc.
Salt Sol. B**		5.000 cc.
Water	to	1.000 cc.

pH adjusted to 6.8

*The inorganic salt solutions are the same as previously reported.

To complete the medium the vitamin constituents were added in the following amounts per 100 cc.:

Stock Solution		gamma per 100 cc.
Riboflavin	(100 gamma/cc. in 0.2N acetic acid)	0.2 cc. or 20 gamma
Ca. pantothenate	(" in water)	0.1 cc. or 10 "
Nicotinic acid	(" " ")	0.1 cc. or 10 "
Thiamine HCl	(" " ")	0.1 cc. or 10 "
Pyridoxine	(" " ")	0.1 cc. or 10 "
Biotin	(0.2 gamma/cc. " ")	0.2 cc. or 0.04 "

The basal medium gave excellent results on acid production with L. arabinosus but not with L. casei. Using this medium and omitting

one of the vitamin constituents in turn, it was found that in using L. arabinosus as the test organism no growth occurs on the omission of nicotinic acid or biotin. Some growth was noted on the omission of calcium pantothenate. It shows clearly that L. arabinosus does not require riboflavin, pyridoxine and thiamine. This medium was used with good results for the assay of nicotinic acid and biotin.

The failure to establish growth with L. casei clearly shows that some constituent or constituents for growth is lacking. For the purpose of investigation various substances were used in conjunction with the basal medium. Nucleic acid was added to the medium with the thought in mind that it might supply the required factor as a carbohydrate or a lacking purine or pyrimidine base. Yeast supplements were added to the medium as prepared by Strong, Feeny and Earl[33]. In the addition of the Strong and Carpenter's yeast supplement (equivalent to 20 mgs. of whole autolyzed yeast per 10 cc.) the above medium gave the greatest growth and acid production.

This will give practically a synthetic medium of known chemical composition for microbiological assay. The replacement of the yeast supplement by a factor of known composition would give an entirely synthetic medium of chemically defined composition.

Snell and Peterson[21] found that L. casei required two separate factors for growth obtained from yeast extract by absorption on Norit A. They termed these factors the "filtrate" and "eluate" fractions.

Landy and Dicken[20] report biotin as a growth factor essential for L. casei. They employed the basal medium of Snell and Peterson[31] modified by the replacement of the Norit eluate fraction with folic

acid in excess. They found that biotin would replace the filtrate fraction and produce equivalent growth. They also found that the amount of biotin used by Snell and Peterson[34] was insufficient to support maximum growth. Mitchell, Snell and Williams[36] also report folic acid as a factor stimulating growth for L. casei. These findings suggested the use of folic acid and biotin in place of the yeast extract as a growth factor for L. casei. The formula of the complete medium is given in Table IV. Best results are obtained with this medium when freshly prepared. Heating on a steam bath for a short period aids in the solution of the amino acids. This should be done before the addition of the vitamins. A slight sedimentation and turbidity may remain which can easily be removed by filtration. The amount of glucose and sodium acetate (2%) is double that used in the Snell and Strong medium. These amounts give better results. All vitamin solutions should be freshly prepared. (U.S.P.Reference) Standard riboflavin and thiamin were used in assay. The other vitamins and amino acids were obtained from Merck and Company, Folic acid was kindly supplied by R. J. Williams, Department of Chemistry, University of Texas.

The assay procedures are practically the same as in other microbiological assays. For any one essential vitamin a quantitative response to growth and acid production was found to be consistently proportional to the amount of vitamin supplied. In making the assay for any one vitamin, the vitamin under test is omitted from the basal medium. The basal medium previously mentioned is of double strength and only 5 cc. is required for each culture tube. Assays are carried out in 5/8 x 6

TABLE IV

Complete Medium

Amino acid mixture (Table III)		7.480 gms.
Glucose		10.000 gms.
Sodium Acetate		10.000 gms.
Salt Soln. A		5.0 cc.
Salt Soln. B		5.0 cc.
Riboflavin (100 gamma/cc. in 0.2N acetic acid)		2.0 cc.
Biotin (Free acid) 0.5 gamma/cc. in Water		10.0 cc.
Cal. Pantothenate (100 gamma/cc.)		2.0 cc.
Nicotinic acid (100 gamma/cc.)		2.0 cc.
Soln. Pyridoxine (100 gamma/cc.)		4.0 cc.
Soln. Thiamine (100 gamma/cc.)		1.0 cc.
Soln. Folic acid (10 gamma/cc.)		1.0 cc.
Distilled Water	to	500 cc.

pH adjusted to 6.8

inch culture tubes. Duplicate tubes are set up in the range of concentration required as standard. Concentrations of the vitamin in question in amounts to meet the required range are added to the culture tubes and brought to a volume of 5 cc. with distilled water. (The solution must be of such a concentration that the highest required amount will be contained in no more than 5 cc.) Five cc. of basal medium is then added to each tube to bring the total volume to 10 cc. In like manner solutions of the sample under test are prepared. The tubes are plugged with cotton and sterilized at fifteen pounds pressure for 15 minutes. After cooling they are planted with a suspension of L. casei and incubated at 37°C. for 72 hours. After incubation the acid production is determined by titration with 0.1 N. NaOH. Values obtained with the standard solutions are set up in a standard curve from which the vitamin content of any dilution of sample may be calculated.

The stock culture is carried in yeast dextrose agar stabs. The original cultures were obtained from the American Type Culture Collection of the Georgetown University Medical School, Washington, D. C. The cultures used were L. arabinosus 8014 and L. casei 7469. The yeast agar stabs are prepared and kept according to the method of Snell & Strong[9]. In place of planting into yeast dextrose broth or the basal medium of Snell & Strong, plantings are made in the basal synthetic medium (Table IV). In this way there can be no carry over of any traces of vitamin content from yeast or casein hydrolysate to interfere with the assay. After 24 hour incubation this culture is centrifuged, the supernatant liquid is decanted and then resuspended in 10 cc. of 0.85 per cent sodium chloride solution. One tenth cc. of this suspension is used for seeding of the assay tubes and culture tubes under test. The basal medium used for seeding should

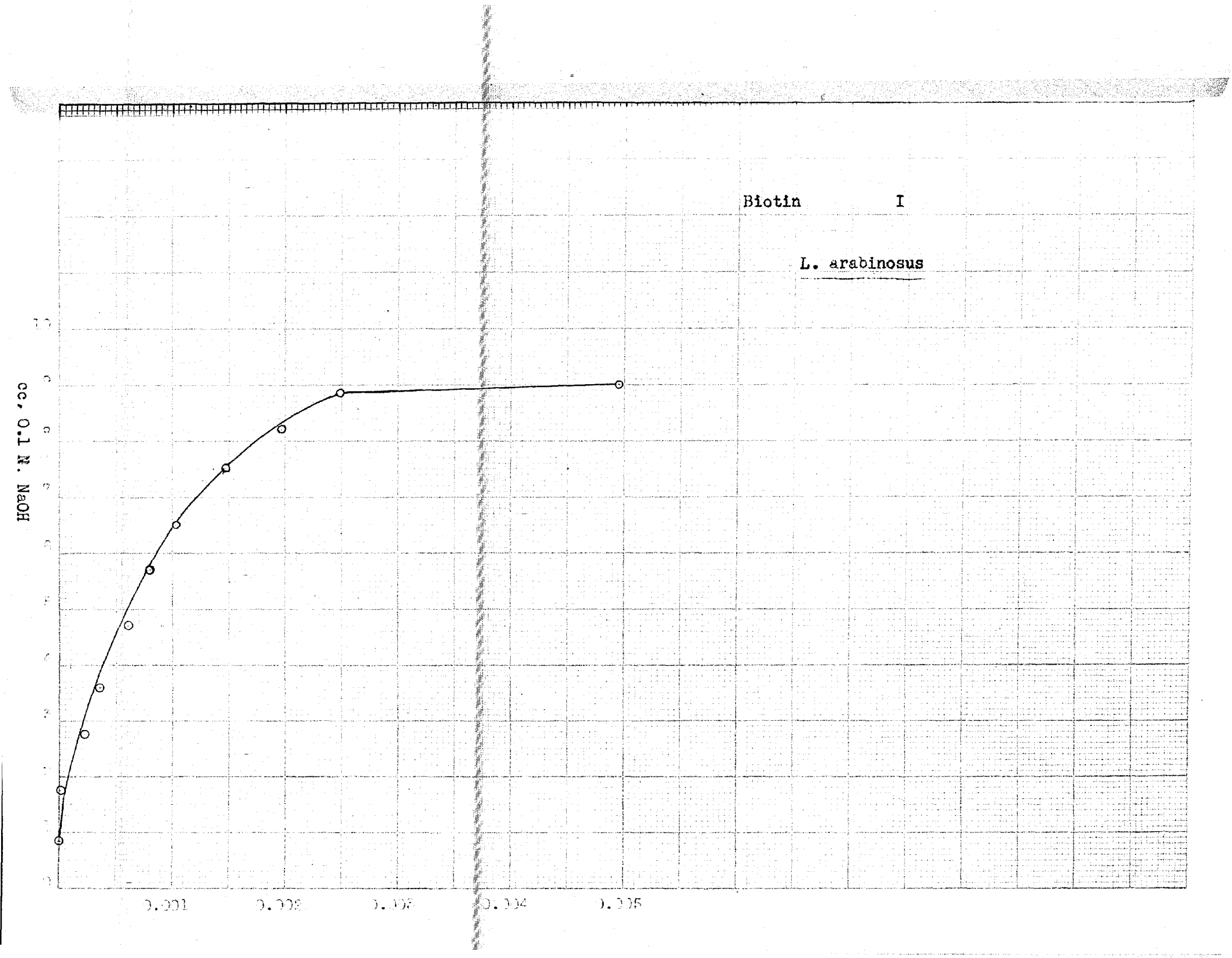
be freshly prepared in order to obtain maximum results. In order to insure the potency and to prevent spoilage the biotin and the folic acid are dissolved in sterile distilled water in the concentrations named, and aseptically transfered to 10 cc. ampules. These ampules are then sealed and used as occasion arises. Care should be exercised in the storage and treatment of the vitamin solutions, especially riboflavin. There should be no undue exposure to light for any length of time. All the vitamin test solutions should be frequently replaced in order to maintain their potency and to prevent decomposition.

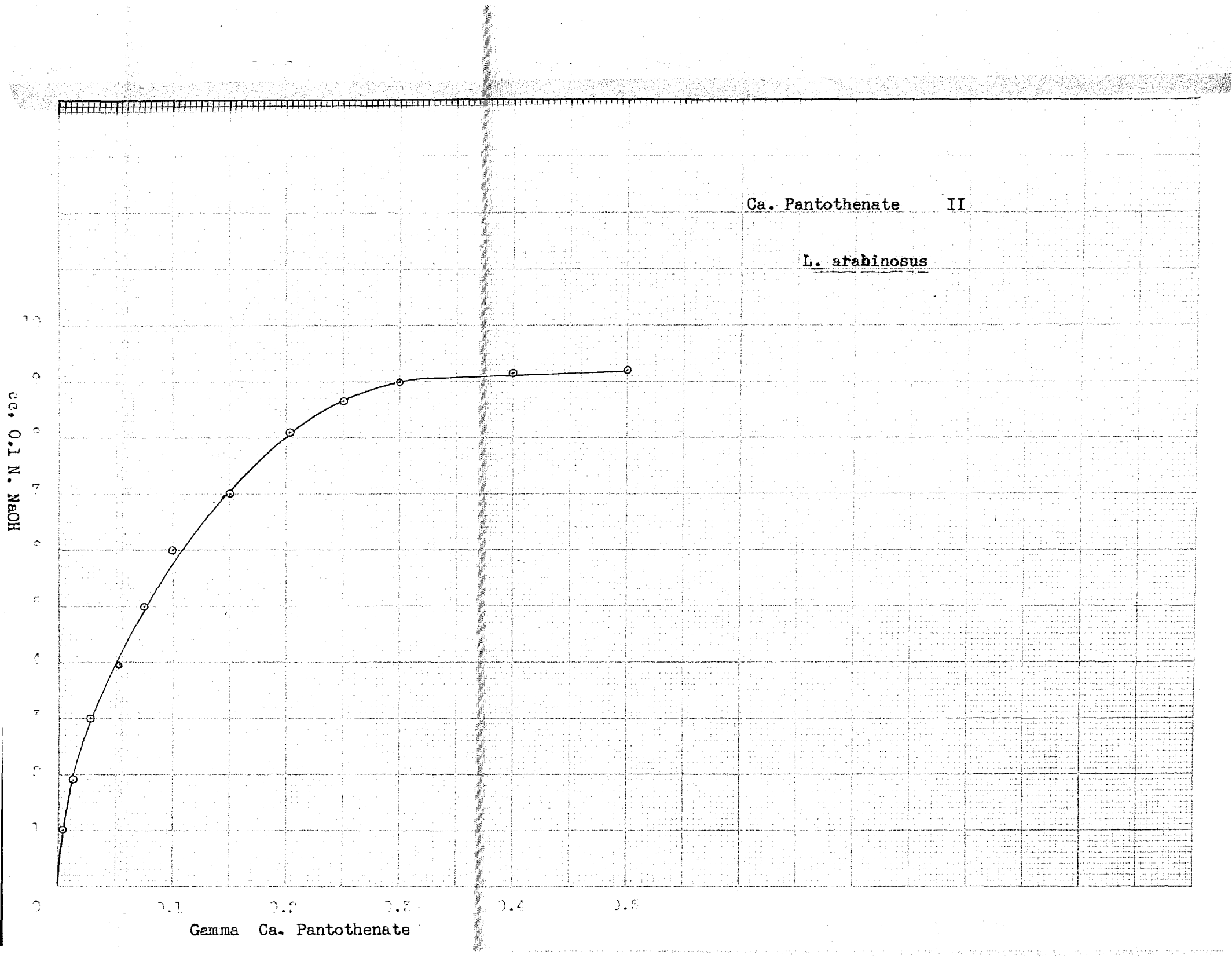
Standard curves for nicotinic acid, biotin and calcium pantothenate result using L. arabinosus according to the following graphs.

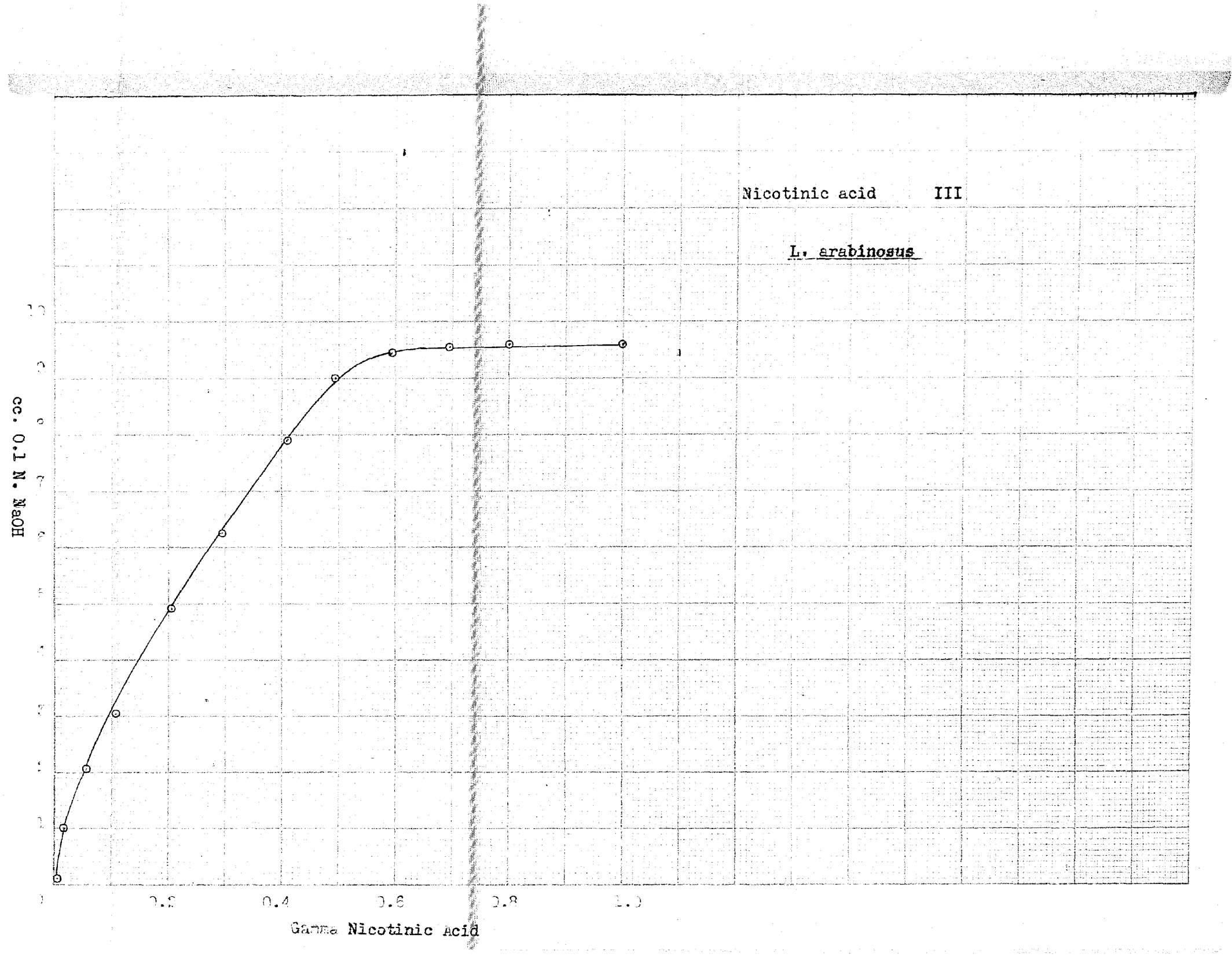
The following standard graphs resulted for riboflavin, Calcium pantothenate, biotin, nicotinic acid, pyridoxine and folic acid, when L. casei was used as the test organism.

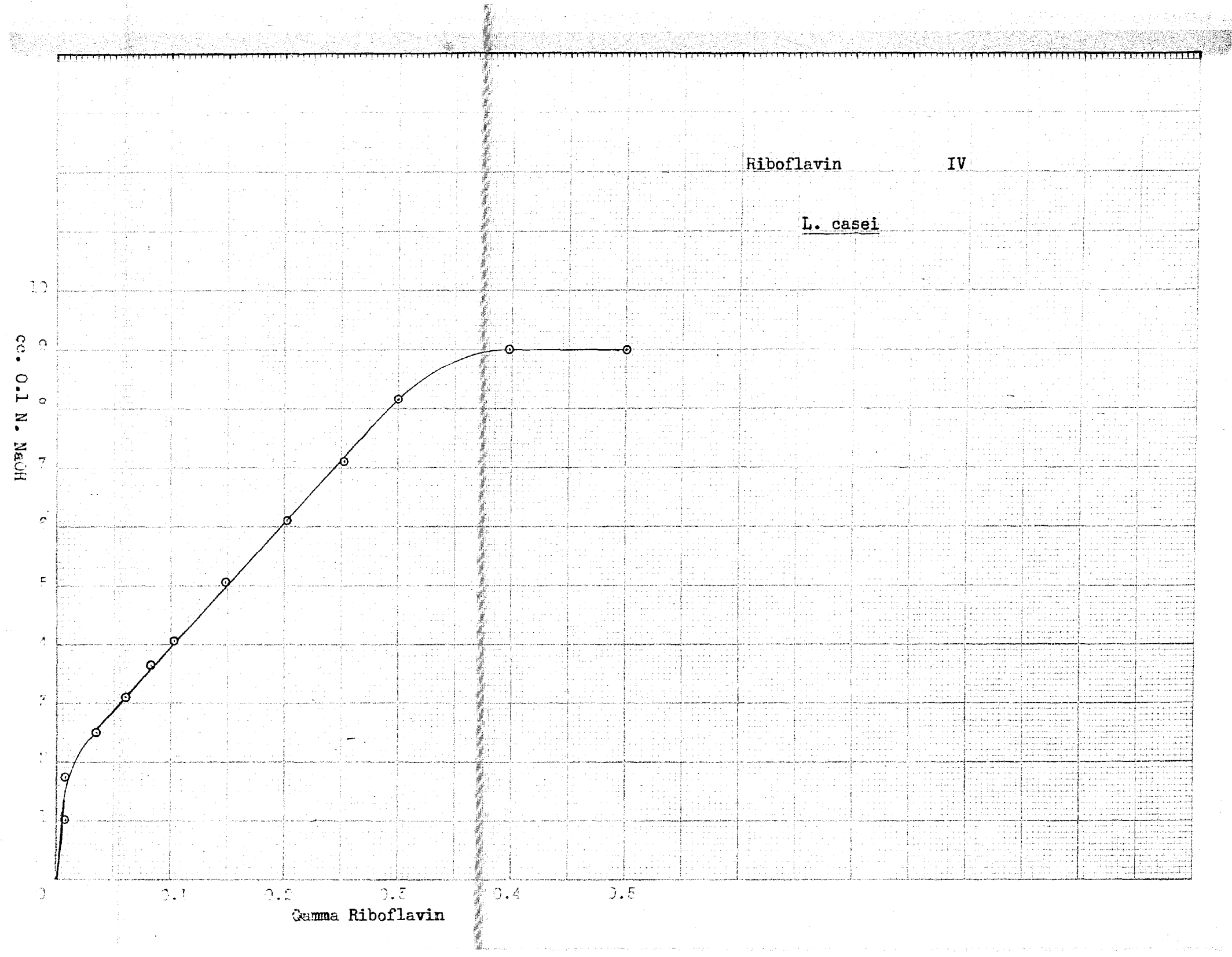
The vitamin content of several products of the Wheatamin Division, De Pree Company, Holland, Michigan was determined by the suggested assay procedure. Results were well in agreement with other established microbiological and spectrophotometric assay methods. A more linear curve appeared to result from the use of this method. In some cases perfect blanks were not obtained; however, blanks equivalent to only 1.0 cc. of 0.1 N acid will not greatly interfere with accurate assay determinations. The products tested were all water soluble and in free form. Products in which the vitamins are in combined form would require special hydrolysis previous to microbiological assay.

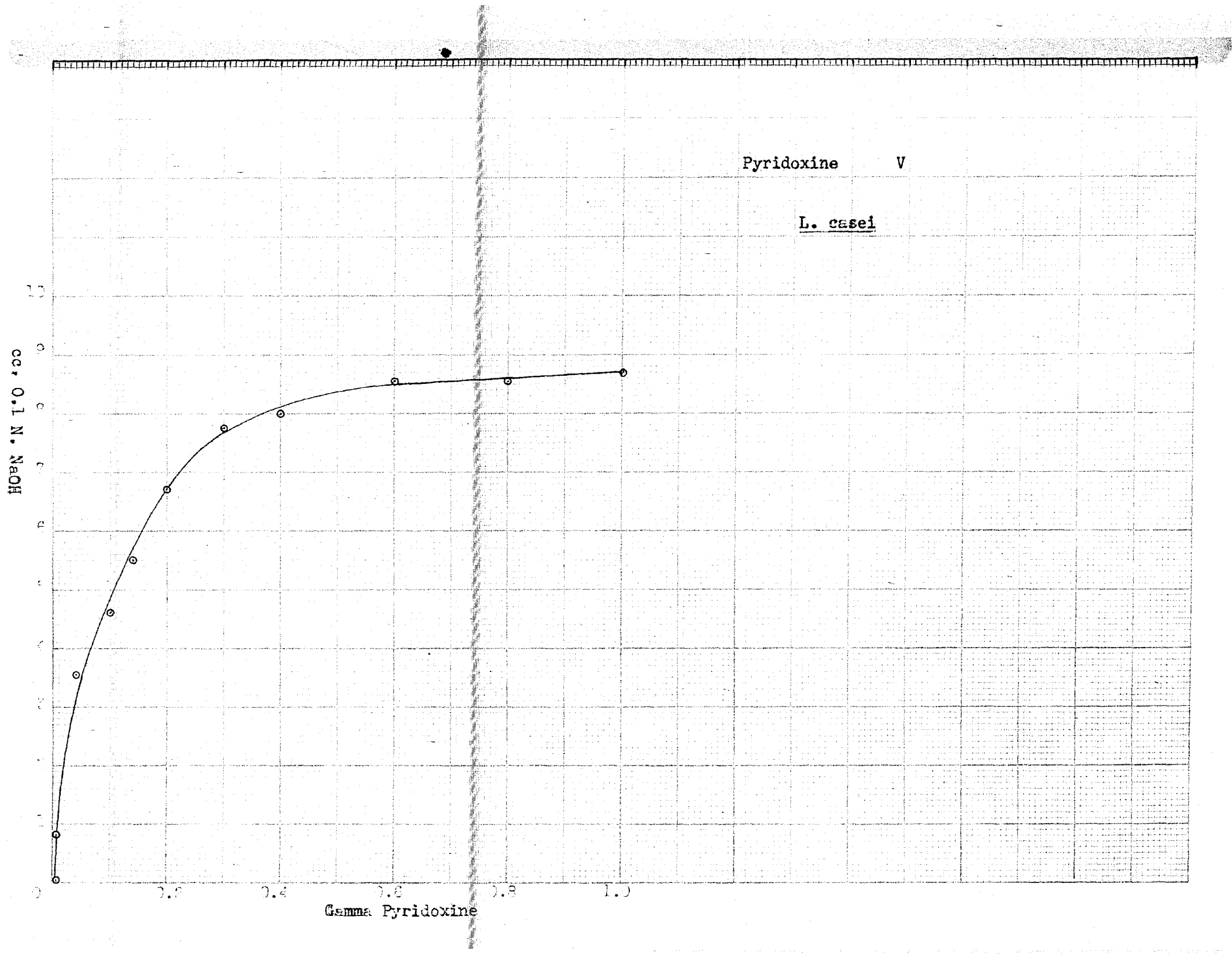
The following graphs, indicate that the procedure is adaptable and practical for assay products of this nature.

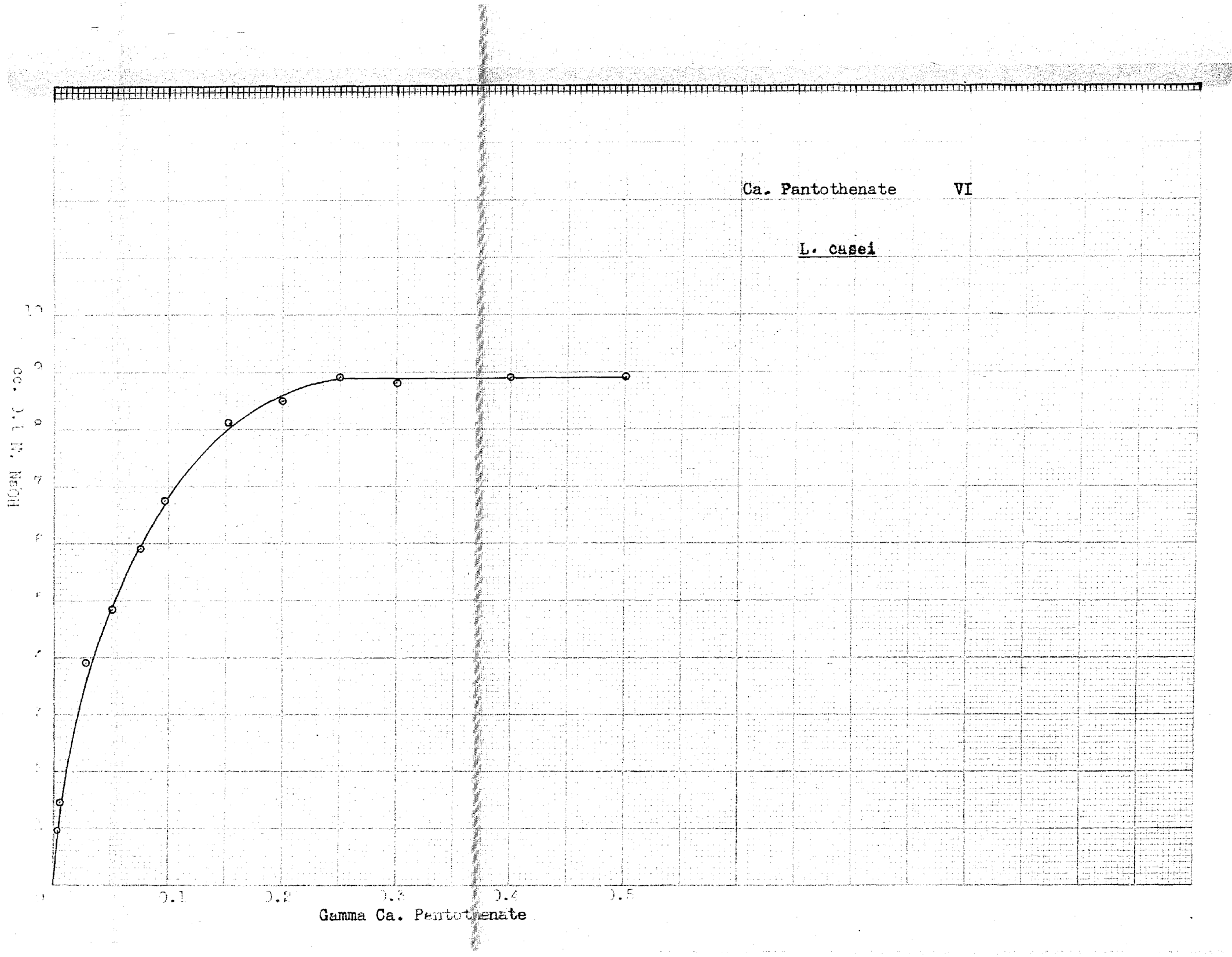

Biotin I
L. arabinosus
cc. 0.1 N. NaOH

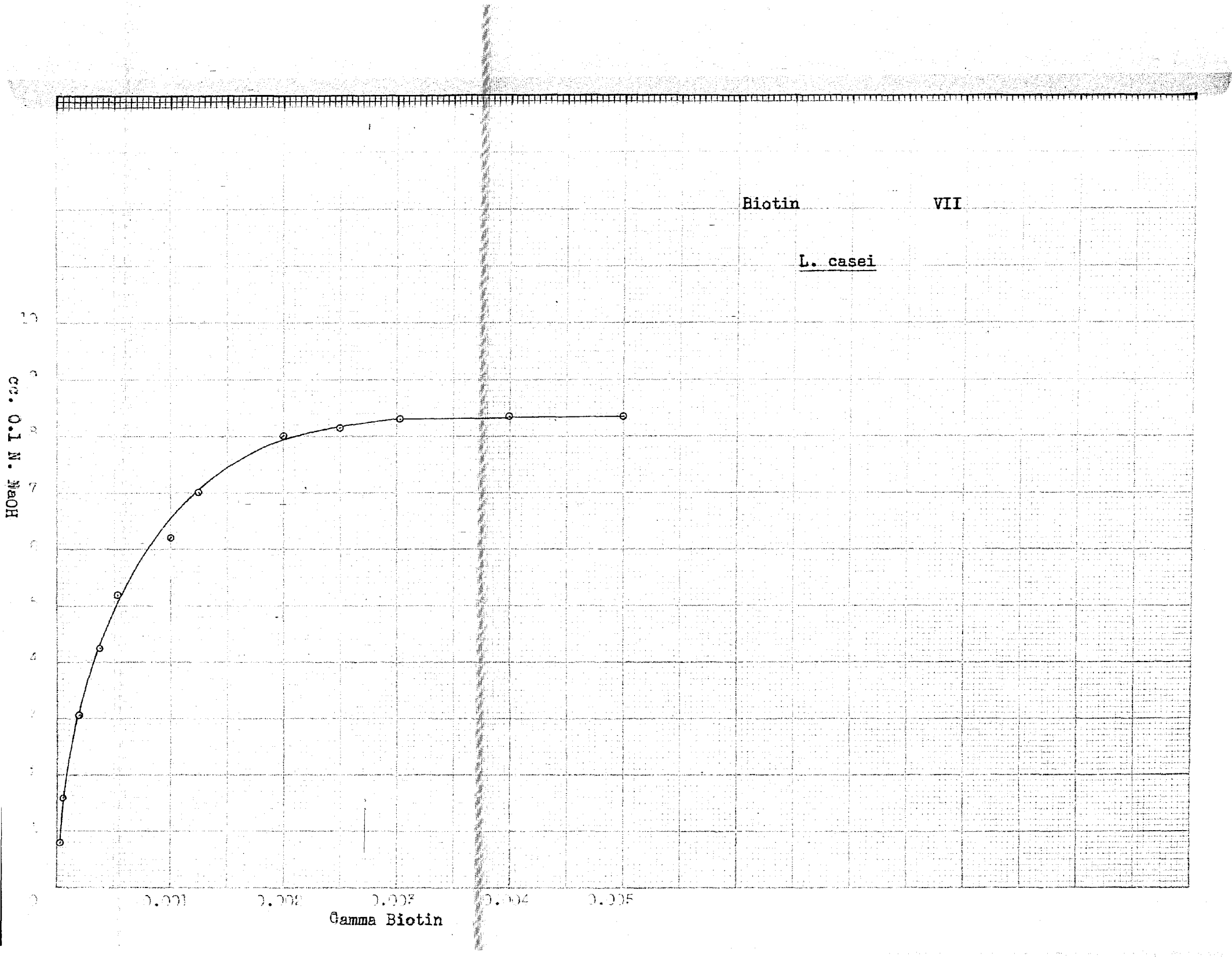

Ca. Pantothenate II
L. arabinosus
cc. 0.1 N. NaOH
Gamma Ca. Pantothenate

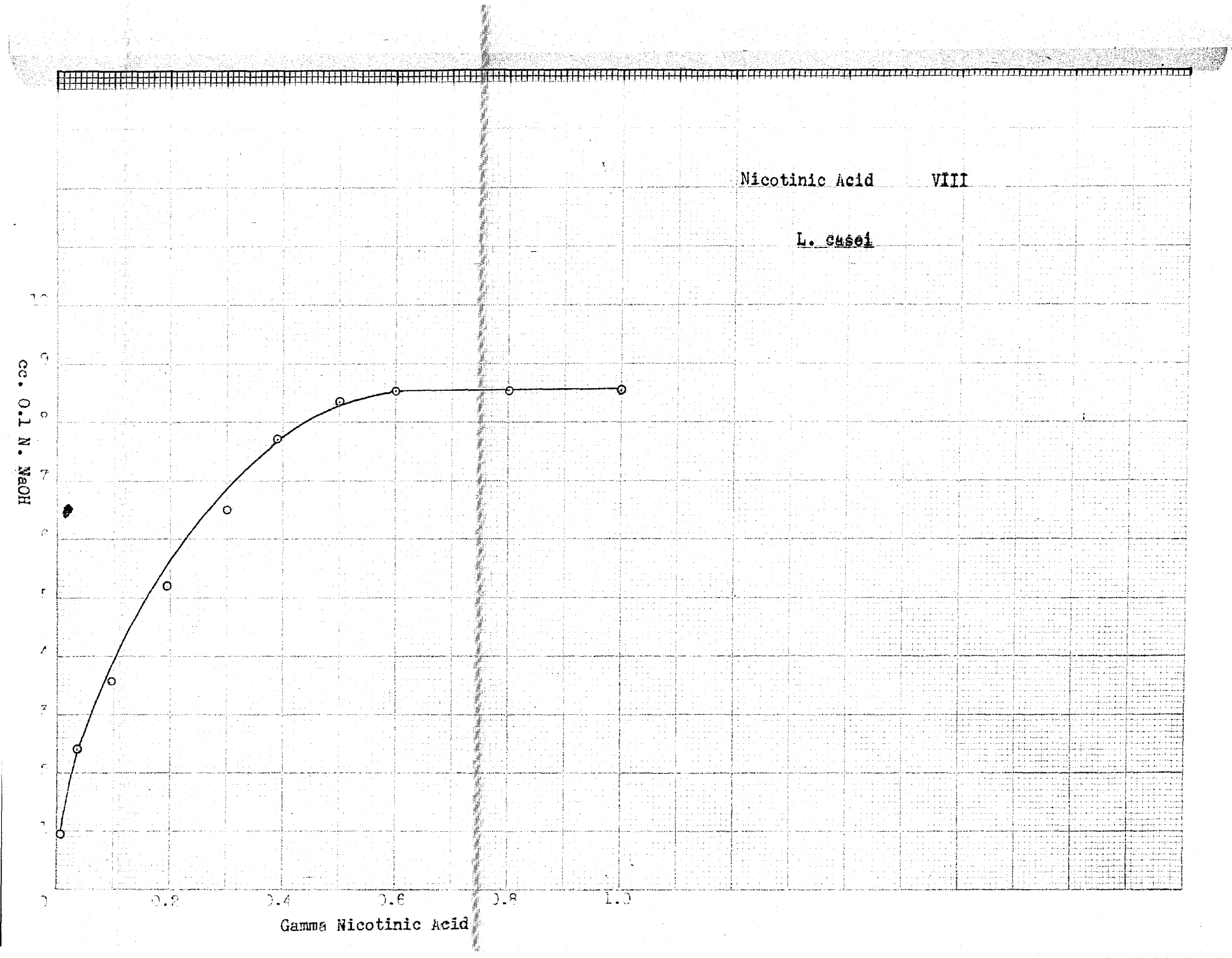

Nicotinic acid III
L. arabinosus
cc. 0.1 N. NaOH
0.2
0.4
0.6
0.8
1.0
Gamma Nicotinic Acid

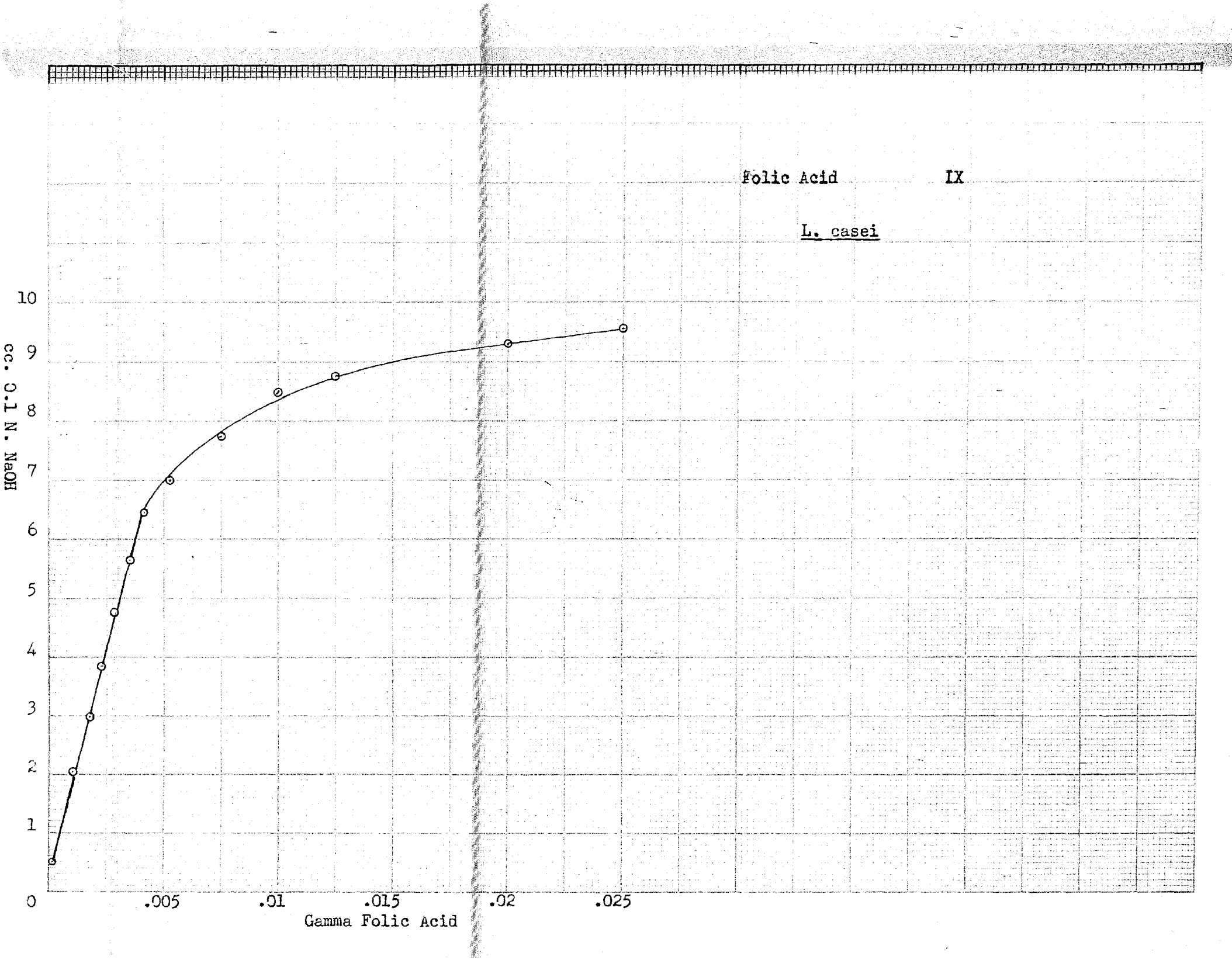

Riboflavin IV
L. casei
cc. 0.1 N. NaOH
Gamma Riboflavin

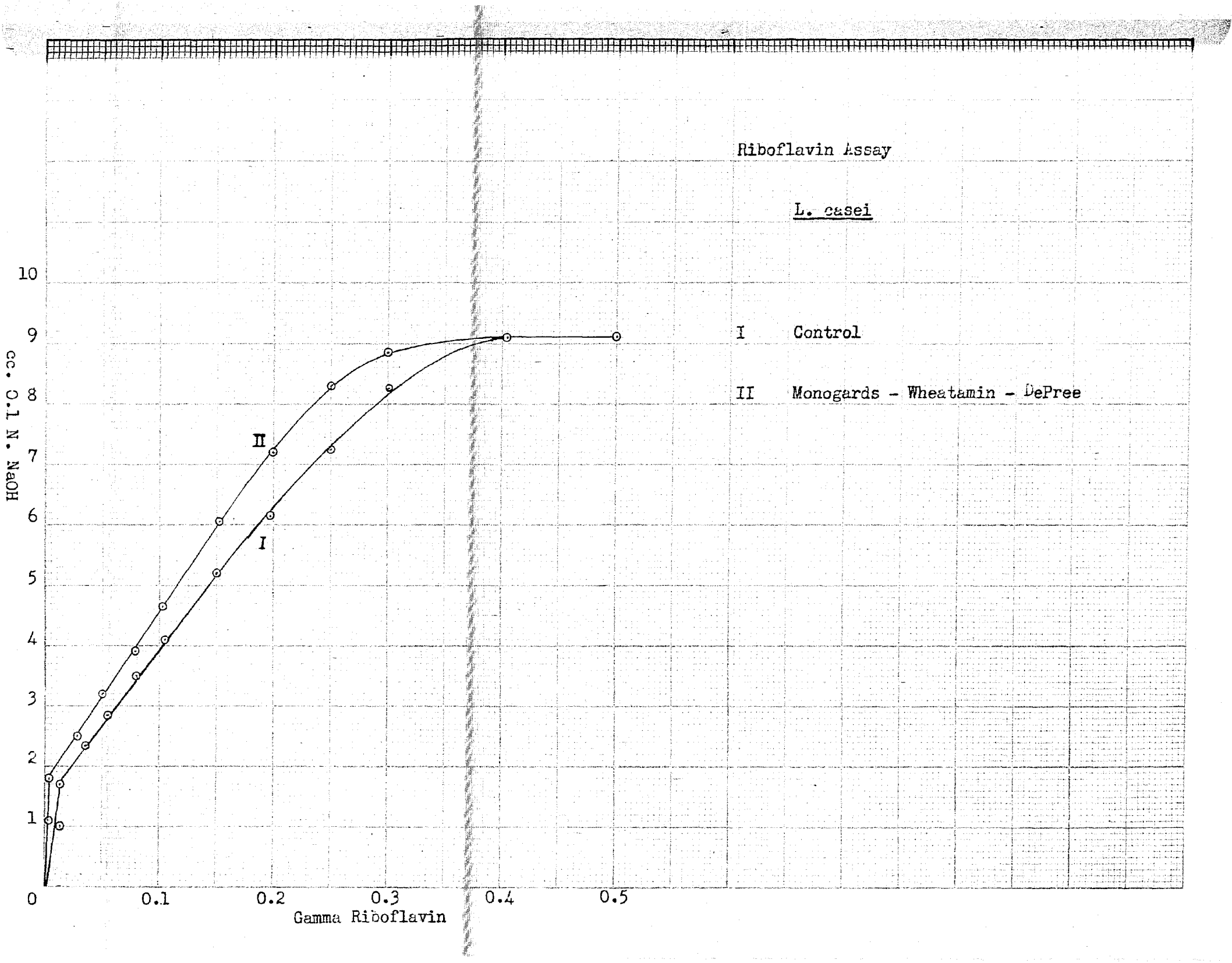

Pyridoxine V
L. casei
cc. 0.1 N. NaOH
Gamma Pyridoxine

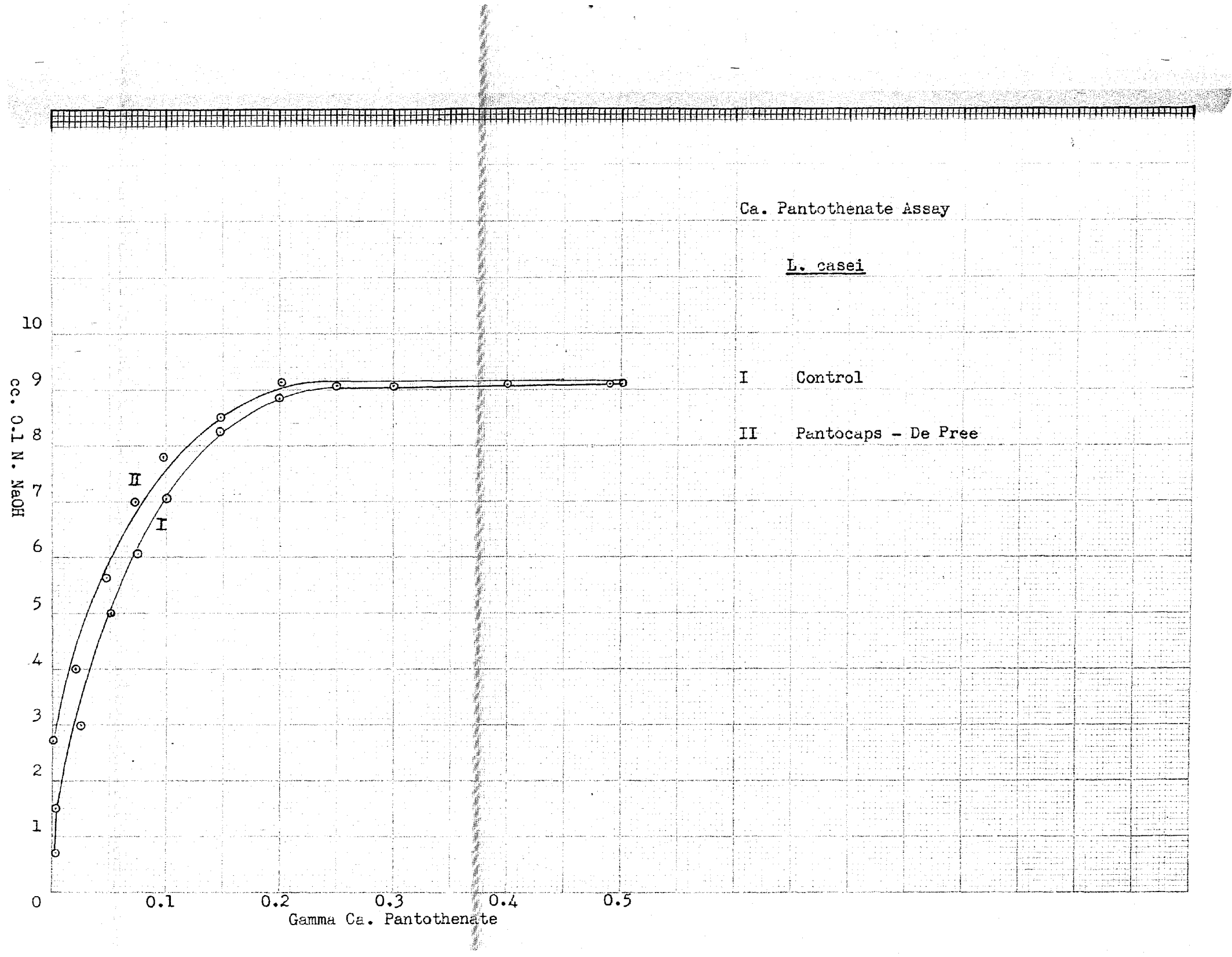

Ca. Pantothenate VI
L. casei
cc. 0.1 N. NaOH
0.1
0.2
0.3
0.4
0.5
Gamma Ca. Pantothenate

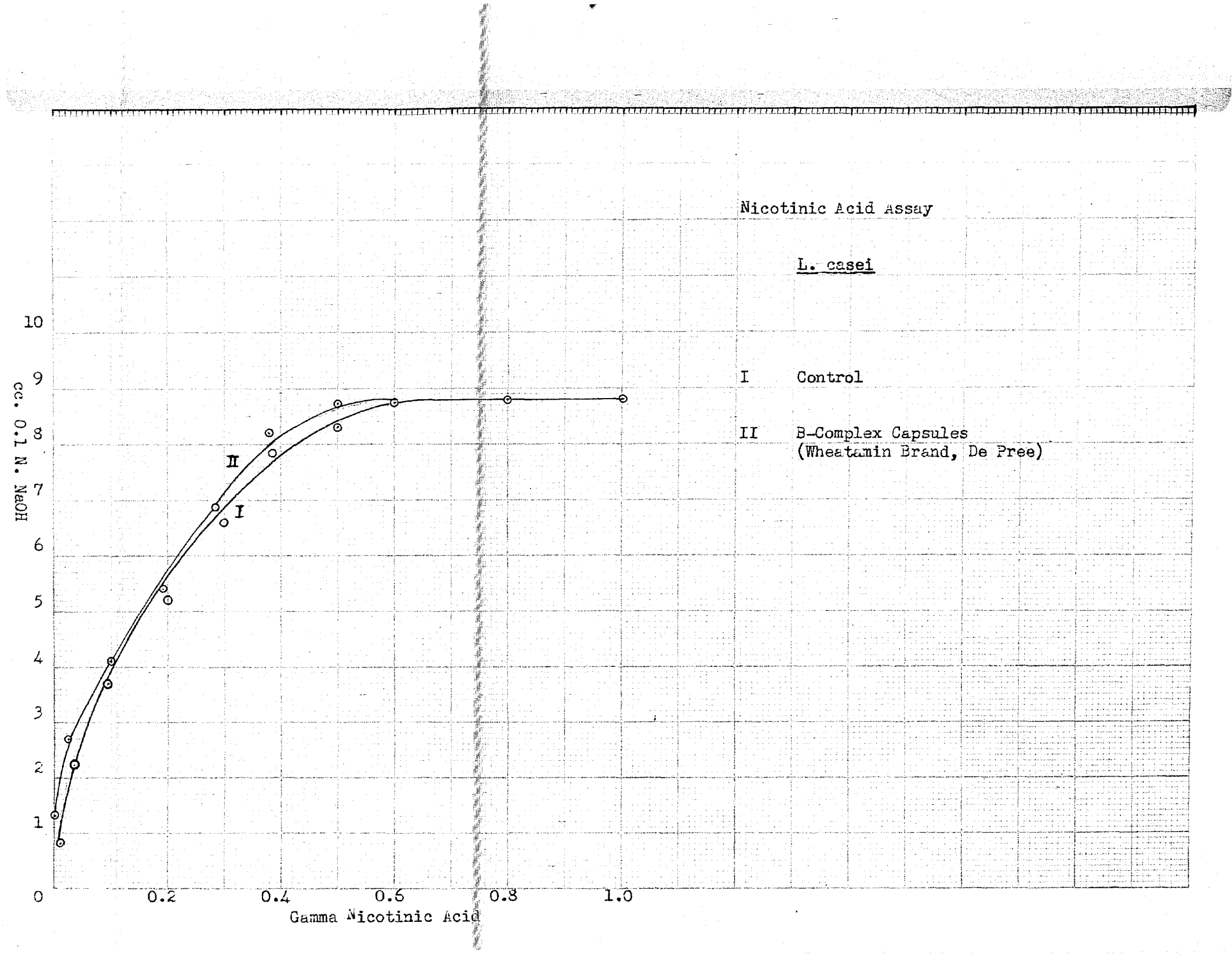

Biotin VII
L. casei
cc. 0.1 N. NaOH
0.001
0.002
0.003
0.004
0.005
Gamma Biotin

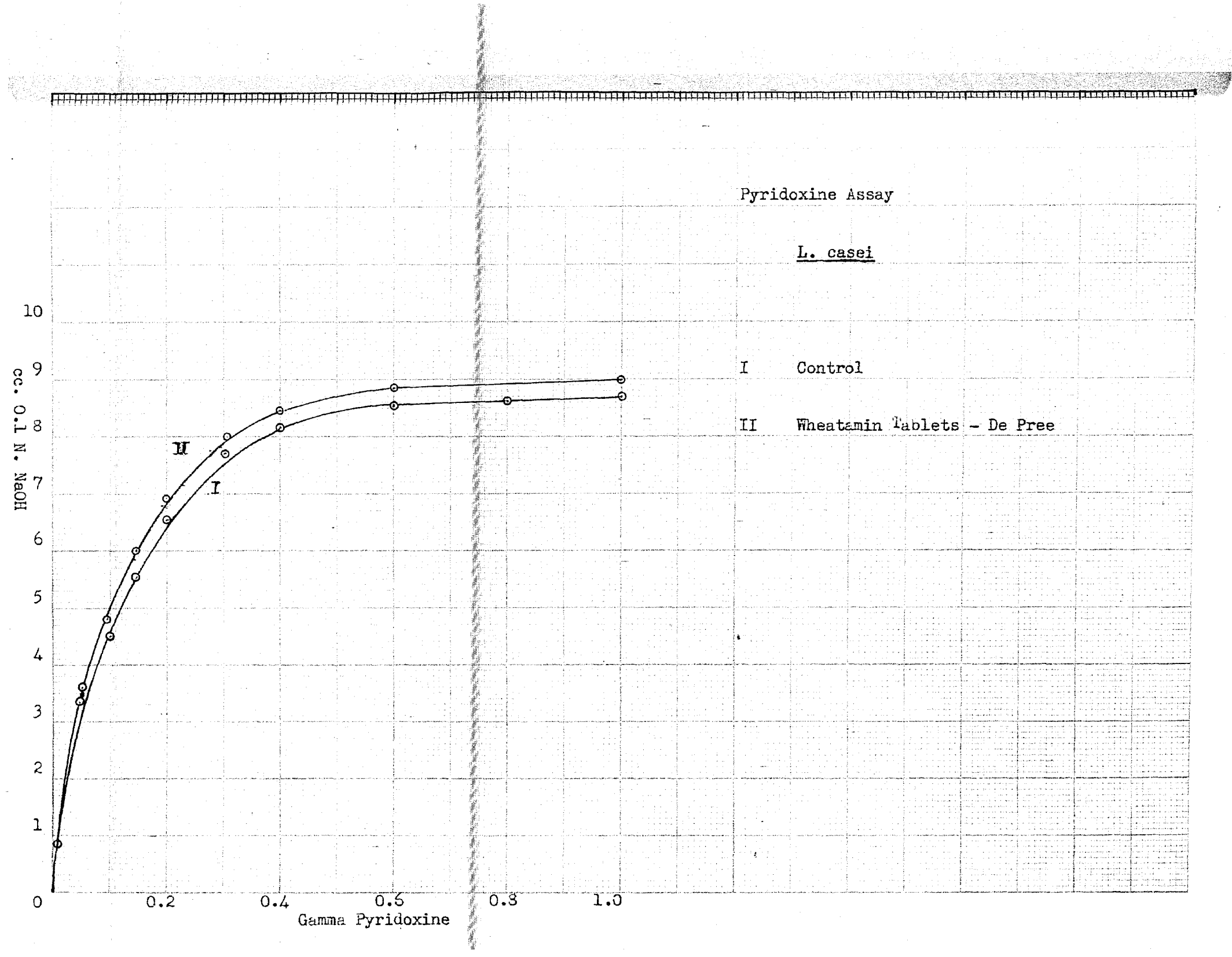

Nicotinic Acid VIII
L. casei
cc. 0.1 N. NaOH
0.2
0.4
0.6
0.8
1.0
Gamma Nicotinic Acid

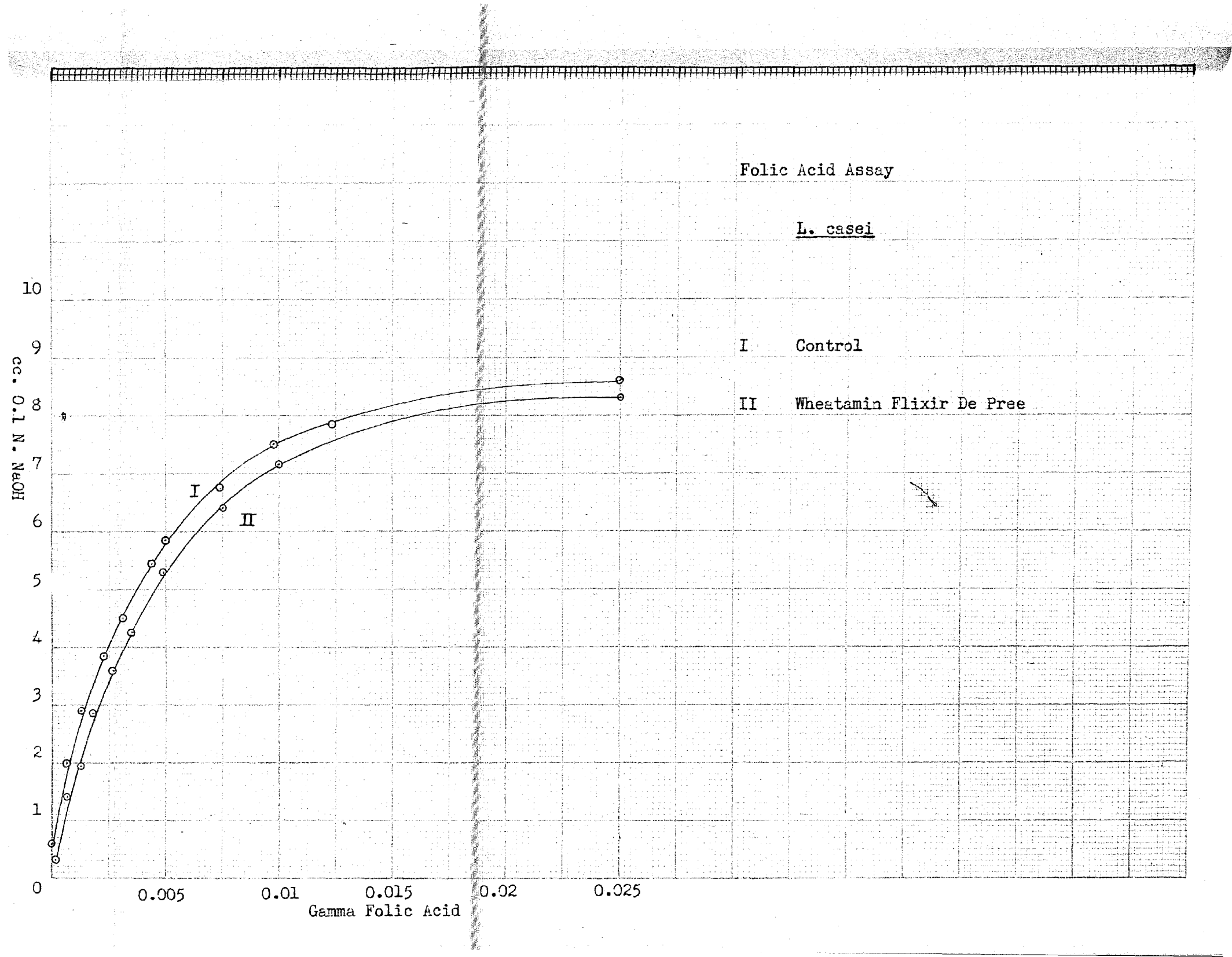

Folic Acid IX
L. casei
cc. 0.1 N. NaOH
10
9
8
7
6
5
4
3
2
1
0
.005
.01
.015
.02
.025
Gamma Folic Acid

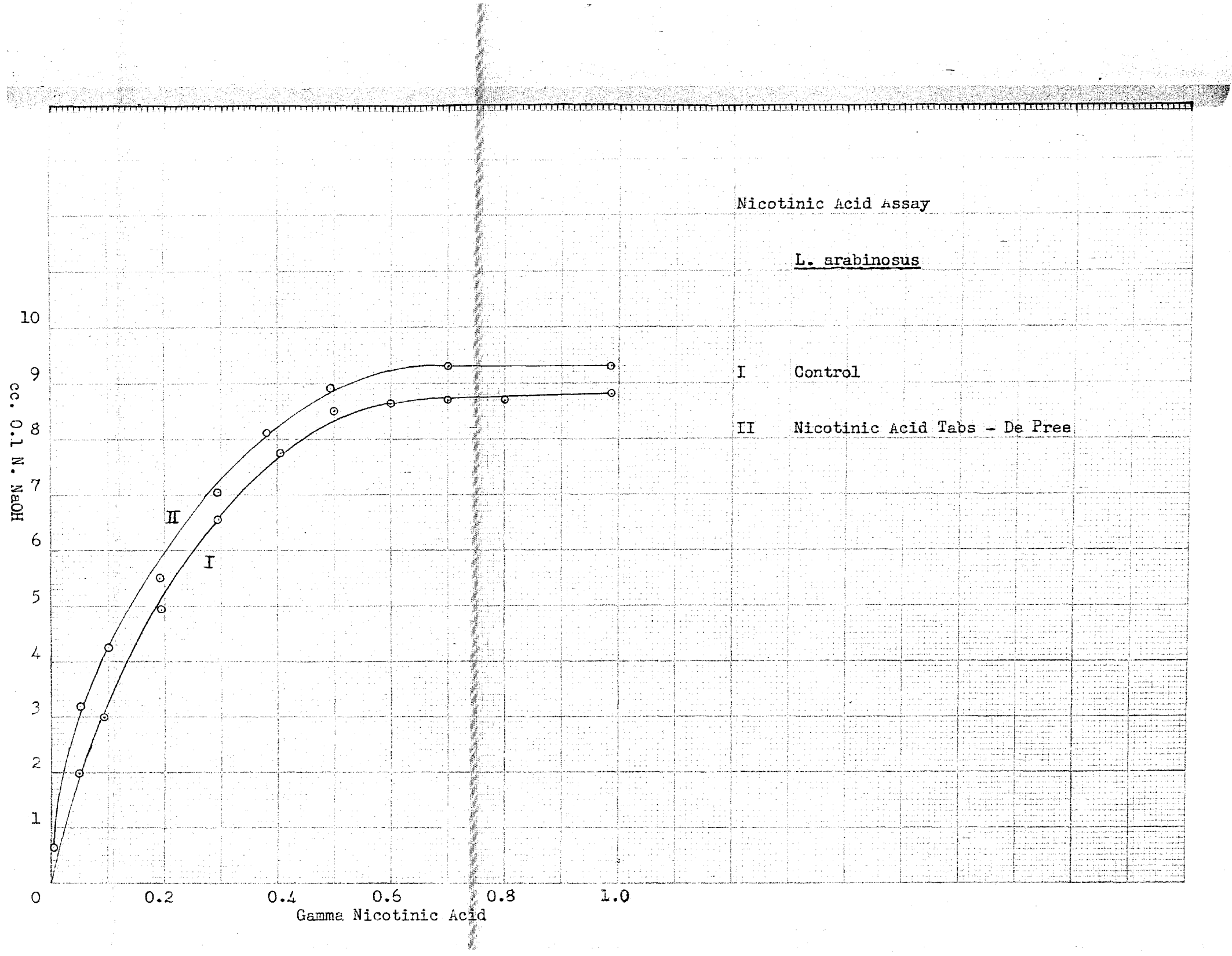

Riboflavin Assay
L. casei
I Control
II Monogards - Wheatamin - DePree
cc. 0.1 N. NaOH
Gamma Riboflavin
0
1
2
3
4
5
6
7
8
9
10
0.1
0.2
0.3
0.4
0.5
I
II

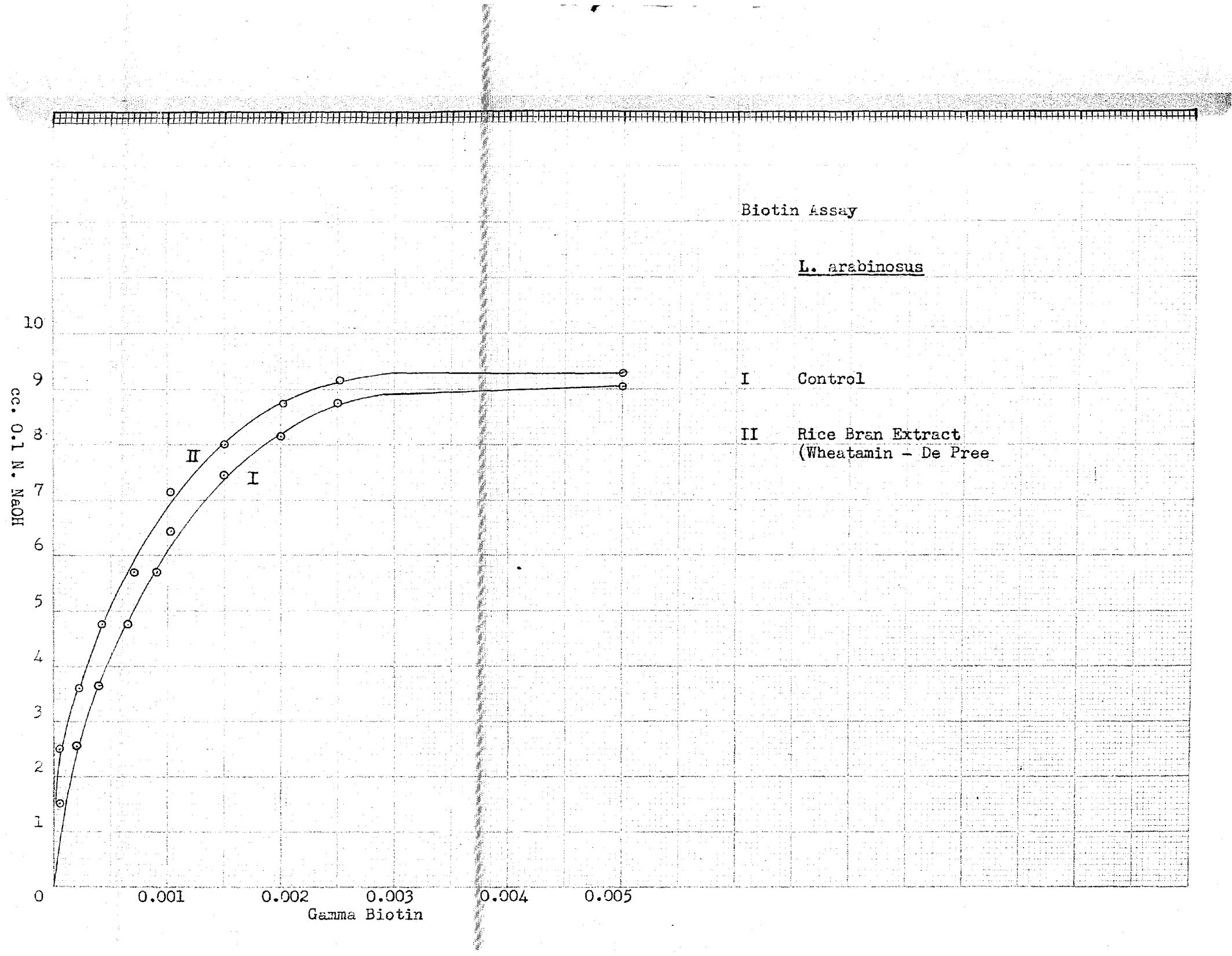

Ca. Pantothenate Assay
L. casei
I Control
II Pantocaps - De Pree
cc. 0.1 N. NaOH
Gamma Ca. Pantothenate
0
1
2
3
4
5
6
7
8
9
10
0.1
0.2
0.3
0.4
0.5
I
II

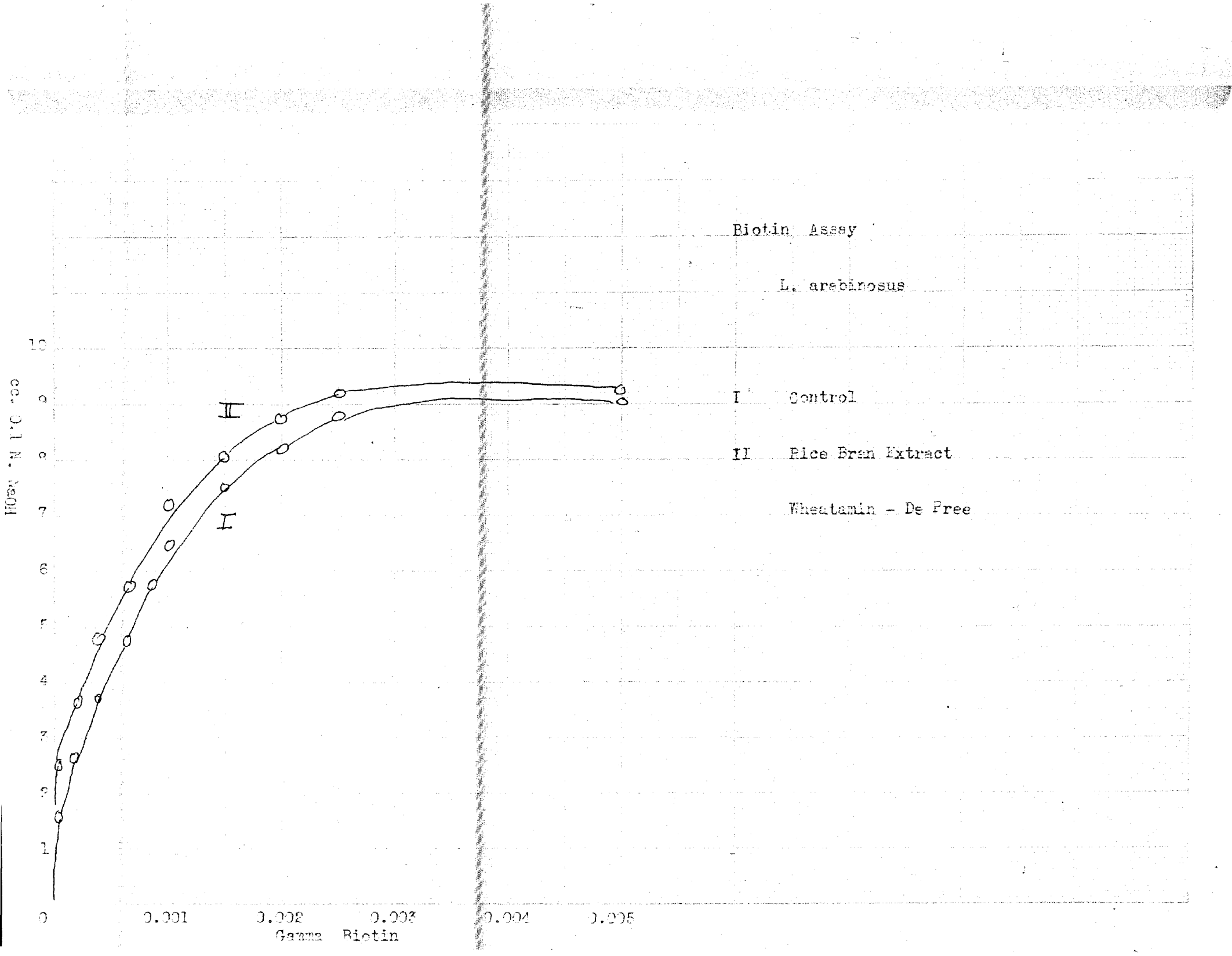

Nicotinic Acid Assay
L. casei
I Control
II B-Complex Capsules
(Wheatamin Brand, De Pree)
cc. 0.1 N. NaOH
Gamma Nicotinic Acid
0
1
2
3
4
5
6
7
8
9
10
0.2
0.4
0.6
0.8
1.0
I
II


Pyridoxine Assay
L. casei
I Control
II Wheatamin Tablets - De Pree
cc. 0.1 N. NaOH
Gamma Pyridoxine
0
1
2
3
4
5
6
7
8
9
10
0.2
0.4
0.6
0.8
1.0
II
I


Folic Acid Assay
L. casei
I Control
II Wheatamin Flixir De Pree
cc. 0.1 N. NaOH
Gamma Folic Acid
0
1
2
3
4
5
6
7
8
9
10
0.005
0.01
0.015
0.02
0.025
I
II


Nicotinic Acid Assay
L. arabinosus
I Control
II Nicotinic Acid Tabs - De Pree
cc. 0.1 N. NaOH
Gamma Nicotinic Acid
0
1
2
3
4
5
6
7
8
9
10
0.2
0.4
0.6
0.8
1.0
I
II


Biotin Assay
L. arabinosus
I Control
II Rice Bran Extract
(Wheatamin - De Pree
cc. 0.1 N. NaOH
Gamma Biotin
0
1
2
3
4
5
6
7
8
9
10
0.001
0.002
0.003
0.004
0.005
I
II


Biotin Assay
L. arabinosus
I Control
II Rice Bran Extract
Wheatamin - De Free
II
I
0.001
0.002
0.003
0.004
0.005
Gamma Biotin

DISCUSSION

The amino acids essential for growth and acid production of the lactic acid bacteria have been demonstrated through careful study. It has been shown that the amino acids, in the combination and quantities shown, are more efficient than the regular hydrolysates of casein in microbiological assays. Determinations were made by a detailed process of addition and elimination. More than five hundred combinations of the amino acids have been investigated. The simplest combination that will produce a good growth consists of the following: 9 amino acids; tryptophane, cystine, glutamic acid, valine, tyrosine, theonine, leucine, isoleucine and arginine. Greater acid production and higher growth levels are attained by the use of all the amino acids shown in Table III.

No resultant growth occurs on the omission of any one of the following amino acids; tryptophane, cystine, glutamic acid or valine, and suggests the possibility of microbiological detection and determination of these acids.

It was found that the omission of riboflavin, pyridoxine, thiamine and folic acid had very little effect upon the growth and acid production of L. arabinosus, while the omission of nicotinic acid, pantothenic acid and biotin resulted in no growth. The omission of any one of these six factors above resulted in no growth response for L. casei. This would indicate that L. arabinosus does not require riboflavin, pyridoxine or folic acid but that L. casei does require all six of the vitamin factors as riboflavin, pantothenic acid, biotin, nicotinic acid, pyridoxine and folic acid.

No results were obtainable in the case of thiamine. A microbiological assay of this factor is difficult due to the fact that it is easily destroyed and will not stand autoclaving at 15 pounds pressure for 15 minutes. Assay of this factor may be readily accomplished by spectrophotometric measurements using the thiochrome method with the adaptation of the Hennessey and Cerecedo procedure.

Preceding graphs show that growth and acid production are proportional to the concentration of the vitamin under test. The amount of vitamin necessary for growth varies with each vitamin. Only very minute amounts are required in each case and the tests are very sensitive. Parallel comparison with results of other methods of the vitamin assays for the various factors now in use are in close agreement. A slight variation in the extreme upper and lower parts of the curve may occur; however, that portion of the curve which is linear is reliable and only can be used for the calculation of assay values. In the experimentation throughout this paper all tests were run in triplicate in order to obtain reliable checks.

The use of Leuconostoc mesenteroides as a test organism was also tried. In some instances it gave promise that it could be used but results were not entirely satisfactory when used in combination with the proposed medium. L. casei gave by far the most satisfactory results. L. arabinosus can only be used for three of these factors.

The procedure has been greatly simplified by the use of the amino acid combination. The time and effort required for the hydrolysation of casein has been removed and the results are more consistent. The use of the yeast supplement also has been done away with. While we have no figures on the cost differential, the expense incurred should not greatly

exceed the cost of a medium using hydrolyzed casein and yeast extract. The ease of preparation would far offset any slight increase in cost.

CONCLUSION

A medium has been produced consisting of entirely known chemical composition. The procedure has been greatly simplified by the use of the premixture of amino acids and other required compounds. L. casei may be used for the six vitamin assays. Results are more accurate and reliable as the interferences naturally present in hydrolysates of casein yeast, and peptone are not present. The medium presents a simple procedure using known chemical compounds and a single test organism for the assay of the six most important vitamins of the B complex.

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