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THE USE OF CLOSTRIDIUM PERFRINGENS BP6K FOR ANALYZING CASEIN FOR ISOLEUCINE, LEUCINE, METHIONINE PHENYLALANINE, AND VALINE

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THESIS

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THE USE OF CLOSTRIDIUM PERFRINGENS EP6K FOR ANALYZING CASEIN FOR ISOLEUCINE, LEUCINE, METHIONINE PHENYLALANINE, AND VALINE

By

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INTRODUCTION

Microbiological methods for the estimation of amino acids have become important analytical tools in the field of protein analysis. Application of these methods in experimental nutrition has made possible a more detailed knowledge of amino acid assimilation and metabolism. When microbiological methods are properly worked out and carefully controlled, they are capable of yielding results which compare favorably in accuracy with those from the best chemical methods (Snell, 1945).

The advantages and limitations of microbiological methods have been discussed by Snell (1945) and by Schweigert and Snell (1947). All the methods are similar in procedure, well suited to routine use, and demand only the ordinary laboratory equipment. They are highly specific and unusually sensitive. Neither separation of the protein portion of the sample nor separation of the amino acids after hydrolysis is necessary. Incomplete knowledge of the intimate nutrition, the metabolism, and the adaptation abilities of the test organisms is the most important disadvantage of these methods. The inhibitive effect of the unnatural isomers of the amino acids may introduce serious error (Prescott, et al., 1949; Kobayashi, Fling, and Fox, 1948; Fling and Fox, 1945; Fox, Fling, and

Bollenback, 1944).

The lactic acid bacteria are the most widely used group of organisms for microbiological determinations at the present time. Historically they were among the first organisms to be applied to assay procedures, because more was known of their nutritional requirements than of any other group. Snell (1948) attributes the widespread use of the lactic acid bacteria to "the complex nature of their nutritional requirements, their rugged nature, and the fact that their growth could be easily followed by turbidimetric means or by titration of the lactic acid produced during growth". The organisms most commonly used are Lactobacillus arabinosus 17-5, Lactobacillus casei Bc-1, Lactobacillus fermenti 36, Streptococcus faecalis, and Leuconostoc mesenteroides.

Other organisms have been suggested for assay purposes. "Leucineless" mutants of Neurospora crassa have been used for the estimation of leucine by Ryan and Brand (1944) and by Hodson and Krueger (1947). Doermann (1945) also has used a mutant of Neurospora for the estimation of lysine. Tryptophane has been estimated by means of Eberthella typhosa T 63 by Wooley and Sebrell (1945). Escherichia coli has been recommended (Roepke, Libbey, and Small, 1944), but details of the method used have not yet appeared.

A microbiological procedure for assay of amino acids with Clostridium perfringens EP6K has been published recently (Boyd, Logan, and Tytell, 1948). It is the purpose of this study to compare this new method with a standard method using Lactobacillus arabinosus 17-5 by analyzing casein for isoleucine, leucine, methionine, phenylalanine, and valine.

REVIEW OF THE LITERATURE

REVIEW OF THE LITERATURE

The first application of lactic acid bacteria to quantitative assay work was made by Snell and Strong (1939) for the estimation of riboflavin with Lactobacillus casei. Most of the basic techniques employed in all microbiological determinations today are derived from this early vitamin assay method.

One of the first microbiological methods for the determination of amino acids utilized Lactobacillus arabinosus 17-5 (Shankman, Dunn, and Rubin, 1943). Much of the pioneer work on amino acid assay has been done with Lactobacillus arabinosus and it is now one of the most commonly used lactic acid bacteria for assay procedures. A Lactobacillus arabinosus method has been chosen as the standard method in this study. The following discussion, therefore, has been limited to those references pertaining to the development and use of microbiological procedures utilizing Lactobacillus arabinosus and the Clostridium perfringens method being tested.

Snell and Wright (1941) were the first workers to subculture Lactobacillus arabinosus successfully on a completely synthetic medium (a mixture of amino acids as the main source of nitrogen).

The actual amino acid requirements of Lactobacillus arabinosus have been determined independently

by Shankman (1943), Kuiken et al. (1943b), Hegsted (1944) and Dunn et al. (1947). All the workers agree on the essential nature of glutamic acid, isoleucine, leucine, tryptophane, and valine for growth of the organism and on the non-essential nature of glycine, hydroxyproline, norleucine, and norvaline. The latter, however, were declared to be necessary for optimal growth. There is disagreement between workers as to whether arginine, lysine, phenylalanine, threonine, and tyrosine are essential or accessory growth factors.

This failure to agree completely has been attributed to the impurity of some commercial sources of amino acids (Hegsted and Wardwell, 1944), the composition of the medium (Brickson et al., 1948; Stokes and Gunness, 1945), and the possibility of variation in the test organism itself. Dunn et al. (1947) has demonstrated quantitative differences in acid production by six cultures of Lactobacillus arabinosus.

It is generally assumed in microbiological assays that only the L form of the amino acids are utilized by the test organisms. Kuiken et al. (1943a, 1943b) have submitted proof that only the natural isomers of isoleucine, leucine, glutamic acid, lysine, and valine are active for Lactobacillus arabinosus. Stokes and Gunness (1944) have reported that the naturally occurring enantiomorphs of threonine and

methionine are the only ones available to Lactobacillus arabinosus. Although Hegsted (1945) found some activity for the D forms of leucine, isoleucine, and valine, he concluded that the error caused by the use of DL amino acids as standards in ordinary assays would be small.

In microbiological assay procedures, total growth permitted by given concentrations of the essential substances is measured rather than comparative rates of growth. Determinations of terminal growth after prolonged incubation have been found to yield more reliable results than determinations made before growth has gone to completion (Snell, 1945). The incubation period which will allow the maximum possible response of Lactobacillus arabinosus and other lactic acid bacteria is 72 hours. This three day period is a disadvantage in the practical application of Lactobacillus arabinosus methods, for it imposes a limit on the number of determinations that can be performed routinely and thus increases the time necessary to complete a given analysis.

Table I summarizes the amino acids which have been determined with Lactobacillus arabinosus by various workers.

Table I

Investigators Who Have Used

Lactobacillus Arabinosus 17-5 for the

Analysis of Various Amino Acids

Amino Acids	Investigators
Glutamic acid	Dunn, et al. (1944) Lyman, et al. (1945) Lewis and Olcott (1945) Hao, et al. (1945) Hier, et al. (1945) Baumgarten, Mather, and Stone (1945) Henderson and Snell (1948)
Isoleucine	Kuiken, et al. (1943b) Schweigert, Tatman, and Elvehjem (1945) Hier, et al. (1945) Baumgarten, Stone, and Boruff (1945) Barton Wright (1946)
Leucine	Kuiken, et al. (1943b) Schweigert, et al. (1944) Hier, et al. (1945) Baumgarten, Stone, and Boruff (1945) Barton Wright (1946) Camien and Dunn (1948) Henderson and Snell (1948)
Methionine	Dunn, et al. (1946) Horn, Jones, and Blum (1946) Riesen, Schweigert, and Elvehjem (1946)
Phenylalanine	Hegsted (1944) Henderson and Snell (1948)
Tryptophane	Greene and Black (1944) Schweigert, Sauberlich, and Elvehjem (1945) Dunn, et al. (1945) Wooley and Sebrell (1945) Henderson and Snell (1948)
Valine	Kuiken, et al. (1943b) Schweigert, et al. (1944) Mc Mahan and Snell (1944) Hier, et al. (1945) Barton Wright (1946) Henderson and Snell (1948)

The growth requirements of Clostridium perfringens BP6K and the microbiological assay procedure using this bacterium were published by Boyd in 1948. As far as the writer knows, no other studies using the Clostridium perfringens as an assay agent have been made.

Thirteen amino acids were found to be absolute requirements for growth of Clostridium perfringens: arginine, histidine, isoleucine, leucine, methionine, threonine, phenylalanine, tryptophane, valine, glutamic acid, serine, cystine, and tyrosine. Those amino acids which appeared to be non-essential for growth were glycine, alanine, lysine, aspartic acid, proline, and hydroxyproline. They were added to the basal medium, however, because of their stimulating effect on growth of the organism.

The amino acids which are essential for the growth of Clostridium perfringens may be assayed with this organism. Lysine also may be assayed if the samples tested do not contain pyridoxamine or pyridoxal.

Maximum growth of Clostridium perfringens under the conditions of the assay procedure is obtained in 16 hours. This short incubation period would be a definite advantage in the application of the Clostridium perfringens assay method to routine analysis of amino acids.

EXPERIMENTAL PROCEDURES

EXPERIMENTAL PROCEDURES

A commercial preparation of casein (Smaco, General Biochemicals) was dried to constant weight at 105 degrees centigrade. Ten samples of 1.000 gram each were weighed into Erlenmeyer flasks and 50 milliliters of two N hydrochloric acid were added. The flasks were plugged with glass wool and then autoclaved for five hours at 15 pounds pressure (Stokes, et al., 1945). When cool, the hydrolysates were filtered and stored in glass stoppered bottles in the refrigerator.

Lactobacillus arabinosus Method

The assay procedure using Lactobacillus arabinosus 17-5 was carried out as described by Schweigert, et al. (1944).

The organism was maintained on stab cultures of yeastextract-dextrose-agar and subcultured weekly. After transfer, the cultures were incubated at 37 degrees centigrade for 48 hours and then held in the refrigerator. The inoculum for the assay tubes was prepared by transfer to the complete basal medium (see Appendix). The inoculum was incubated for 24 hours at 37 degrees centigrade, centrifuged, and the supernatant discarded. The cells were suspended in 20 milliliters of sterile 0.9 percent saline solution. One drop (0.05 milliliters) of this cell suspension was used for the inoculation of each assay tube.

The basal medium was prepared omitting the amino acid under assay, and the pH was adjusted to 6.8 to 7.0 using bromthymol blue as an external indicator. One milliliter of this medium was added to each assay tube. A standard curve of the amino acid which was being determined was obtained by adding graded amounts of that amino acid to a series of tubes. Appropriate dilutions of the casein hydrolysates were made and the pH adjusted to 6.8 to 7.0. Three different dilutions of each test extract were analyzed. The final volume of each tube was adjusted to two milliliters with distilled water.

The racks of test tubes were covered with smooth toweling and autoclaved for 15 minutes at 15 pounds pressure. After cooling, the tubes were inoculated aseptically and incubated in a water bath at 37 degrees centigrade for 72 hours.

The lactic acid produced during growth was titrated with 0.05 N sodium hydroxide, using bromthymol blue as the indicator. A stream of air was introduced into each tube during the titration in order to stir the solution.

Clostridium perfringens Method

The assay procedure for Clostridium perfringens was followed as outlined by Boyd, Logan, and Tytell (1948).

Stock cultures of the organism were maintained in a liquid medium of casein hydrolysate with added tryptophane, cystine, adenine, uracil, vitamins, glucose, salts, phosphate buffer, and defatted beef heart. Serial transfer was made once a week. After transfer, the tubes of stock culture were incubated at 38 degrees centigrade for five to six hours and then stored in the refrigerator.

Seed cultures were grown for inoculum for the assay procedure. The seed culture medium consisted of tryptic digest of casein plus salts and vitamins. Transfers were made from the stock cultures to the seed cultures aseptically. The seed cultures were then incubated for five to six hours at 38 degrees centigrade and stored.

One milliliter of the seed culture was centrifuged and the supernatant discarded. The cells were washed with 10 milliliters of distilled water. The packed cells were then resuspended in one milliliter of water and a dilution of one to 200 was made with water. One drop (0.05 milliliter) of the diluted cell suspension was used for the inoculation of each tube of medium.

The basal medium (see Appendix) which was deficient in the amino acid being analyzed was prepared. The pH was adjusted to 7.1 to 7.2 using phenol red as an external indicator. Five milliliters of this medium were added to each assay tube. To obtain a standard

growth curve, graded amounts of the amino acid under test were added to a series of tubes. The casein hydrolysate samples were diluted according to estimation of the approximate concentration of the amino acid being tested and the pH adjusted to 7.1 to 7.2. Three different dilutions of the hydrolysate were analyzed. The final volume of each tube was made to 10 milliliters with distilled water.

Sodium azide (0.2 milligrams) was added, and the contents of each tube were thoroughly mixed. The tubes were then stoppered with cotton plugs and placed in a boiling water bath for 20 minutes. When cool, they were inoculated and then incubated in a water bath at 45 degrees centigrade for a period of 16 hours.

After incubation, the tubes were mixed by inversion and the contents transferred to matched test tubes*. The density of growth was determined with a Coleman spectrophotometer using a 660 millimicron filter.

The method of calculation of values was the same for both of the assay procedures. Standard curves (see Appendix) were constructed by plotting the concentration of the standard amino acid against the milliliters of 0.05 N sodium hydroxide or percent transmission.

*The tubes were tested for similar light transmittance by comparison in the Coleman spectrophotometer.

The amount of amino acid present in the samples was determined by interpolation of the response of the samples on to this standard curve.

In order to determine the recovery of amino acids, known concentrations of isoleucine, leucine, methionine, phenylalanine, and valine were added to one casein sample. This sample was then submitted to the same treatment of hydrolysis and analysis as the ten test samples of casein.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

The average values obtained for the isoleucine, leucine, methionine, phenylalanine, and valine content of casein are recorded in Table II.

Duplicate samples of each of three dilutions of each casein hydrolysate were analyzed with Lactobacillus arabinosus and with Clostridium perfringens. In microbiological determinations, however, the bacteria in occasional assay tubes fail to grow for unknown reasons. Therefore, six values were not available for each test extract. Inspection of the data showed that one value for each dilution of the hydrolysates was available. When growth was obtained in the duplicate tubes, there was close agreement in the values. It was desirable to have a complete set of values so that the data could be analyzed statistically. Therefore, a selection of values was made according to a definite pattern. The values obtained from the first of the duplicate samples of each dilution of the casein hydrolysates were chosen. If the bacteria had failed to grow in the first of the duplicate tubes, the value obtained from the second of the duplicate tubes was used. Tables of the selected values of the five amino acids may be found in the Appendix.

The average values in grams percent total protein obtained from the Lactobacillus arabinosus

Table II

The Isoleucine, Leucine, Methionine, Phenylalanine, and Valine Content of Casein as Determined by Lactobacillus Arabinosus 17-5 and Clostridium Perfringens EP6K

Amino Acid	Microbiological Method	
	L. arabinosus 17-5	C. perfringens EP6K
	percent	percent
Isoleucine	5.56	5.71
Leucine	9.46	9.71
Methionine	2.79	2.76
Phenylalanine	4.84	5.04
Valine	6.17	6.73

method were isoleucine, 5.56 percent; leucine, 9.46 percent; methionine, 2.79 percent; phenylalanine, 4.84 percent; and valine, 6.17 percent. The average values obtained from the Clostridium perfringens method were isoleucine, 5.71 percent; leucine, 9.71 percent; methionine, 2.76 percent; phenylalanine, 5.04 percent; and valine, 6.73 percent. Differences in the average values for individual amino acids were apparent. Examination of the data, however, revealed differences in the average values of the ten casein hydrolysates in both microbiological methods. The values of the dilutions within the hydrolysates also varied. It was necessary to determine if the variations between casein hydrolysates or the variations from one dilution level to another were greater than the variation in the average values obtained by the use of the two microorganisms. The statistical device employed to separate and evaluate these variations for each amino acid was the analysis of variance.

This statistical analysis showed that the variation between the average values of the casein hydrolysates was not significant in either the Lactobacillus arabinosus method or the Clostridium perfringens method for any of the five amino acids studied. The values obtained for the three dilutions of the casein hydrolysates did not differ significantly in either method for any of the five amino acids.

The F values which were calculated from the data to test the variation of the values obtained by the use of the two organisms are presented in Table III. For isoleucine, leucine, and methionine, the variation of values obtained from the Lactobacillus arabinosus and from the Clostridium perfringens methods were not significant; but the values for phenylalanine and valine did differ significantly.

These results indicate that the Lactobacillus arabinosus method and the Clostridium perfringens method may be used interchangeably under the conditions of this experiment for the analysis of isoleucine, leucine, and methionine. In consideration of the fact that Clostridium perfringens requires a much shorter incubation period than Lactobacillus arabinosus, it probably would be advantageous to use Clostridium perfringens for the routine analysis of isoleucine, leucine, and methionine.

Boyd, Logan, and Tytell(1948) have used Clostridium perfringens for the analysis of the arginine, histidine, isoleucine, methionine, leucine, phenylalanine, threonine, and tryptophane content of β-lactoglobulin, egg albumin, and silk fibroin. Stokes, Gunness, Dwyer, and Caswell analyzed samples of the same preparation of the proteins, using Streptococcus faecalis. Boyd, Logan, and Tytell (1948) stated that "the analytical results obtained by these two different organisms are essentially in agreement". There was, however, one

Table III

F Values Obtained on Comparison of Percentages of Each Amino Acid Measured by Lactobacillus Arabinosus 17-5 and Clostridium Perfringens HP6K

Amino Acid	F Obtained
Isoleucine	0.46
Leucine	3.24
Methionine	2.50
Phenylalanine	9.67**
Valine	28.06**
$F_{.05}$ Required	4.08
$F_{.01}$ Required	7.31

exception to this statement. The value obtained for the phenylalanine content of β-lactoglobulin by the Clostridium perfringens method was 3.2 percent, but the value obtained by the Streptococcus faecalis method was 4.3 percent.

There is no experimental evidence in the present study to explain the significant variation in the values obtained for the phenylalanine and valine content of casein by the use of the two microorganisms. It is possible that one of the organisms is not responding quantitatively to the phenylalanine and valine in the casein hydrolysate. It is also possible that the two organisms are not responding to the same chemical substances.

Satisfactory recoveries of amino acids added to the casein sample before hydrolysis were obtained for both microbiological methods. The percentage recoveries of added amino acids are presented in Table IV. The DL form of isoleucine, methionine, phenylalanine, and valine and the L form of leucine were added to a casein sample for the recovery determinations. The percent recovery of added amino acid was calculated as the response of the organisms to the L form of the amino acid.

The percent recoveries of isoleucine, valine, phenylalanine, and methionine were lower for the Clostridium perfringens method than for the Lactobacillus arabinosus method. In the case of phenylalanine and valine,

Table IV

Percentage Recoveries of Added Amino Acids

Amino Acid	Amount added	Microbiological Methods	
		L. arabinosus	C. perfringens
	mgm.	percent	percent
Isoleucine	25.0	106	98
Leucine	50.0	102	105
Methionine	12.5	105	95
Phenylalanine	12.5	104	99
Valine	25.0	100	98

these lower recovery values yield some interesting information; since they would suggest that Clostridium perfringens was not responding to the D form of phenylalanine and valine. It has been established that Lactobacillus arabinosus utilizes only the L form of phenylalanine and valine (Kuiken, et al. 1943a, 1943b; Hegsted, 1944). Therefore, the higher values obtained by the Clostridium perfringens method for the phenylalanine and valine content of casein were probably not due to the response of the microorganism to the D form of these amino acids.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Ten samples of a commercial preparation of casein were analyzed for isoleucine, leucine, methionine, phenylalanine, and valine by a standard microbiological method using Lactobacillus arabinosus 17-5 and by a new microbiological method using Clostridium perfringens BP6K.

The average values in grams percent of total protein obtained with Lactobacillus arabinosus 17-5 were isoleucine, 5.56 percent; leucine, 9.46 percent; methionine, 2.79 percent; and valine, 6.17 percent. The values obtained with Clostridium perfringens BP6K were isoleucine, 5.71 percent; leucine, 9.71 percent; methionine, 2.76 percent; phenylalanine, 5.04 percent; and valine, 6.73 percent. The variation in the values of isoleucine, leucine, and methionine obtained by the use of the two microorganisms was not significant. The values of phenylalanine and valine as determined by the two methods did differ significantly.

On the basis of these results, it was concluded that the Clostridium perfringens and the Lactobacillus arabinosus methods may be used interchangeably for the analysis of the isoleucine, leucine, and methionine content of casein. The significant differences in the values obtained for phenylalanine and valine by the use of the two microorganisms indicated that

further investigation is needed.

The most important advantage of the Clostridium perfringens method for the estimation of amino acids is the short incubation period required for the maximum growth response of the organism. Clostridium perfringens attains maximum growth in the assay procedure in 16 hours. Lactobacillus arabinosus requires 72 hours in the assay procedure to attain maximum growth. In view of this fact, it would be advantageous to use the Clostridium perfringens method for the routine analysis of isoleucine, leucine, and methionine.

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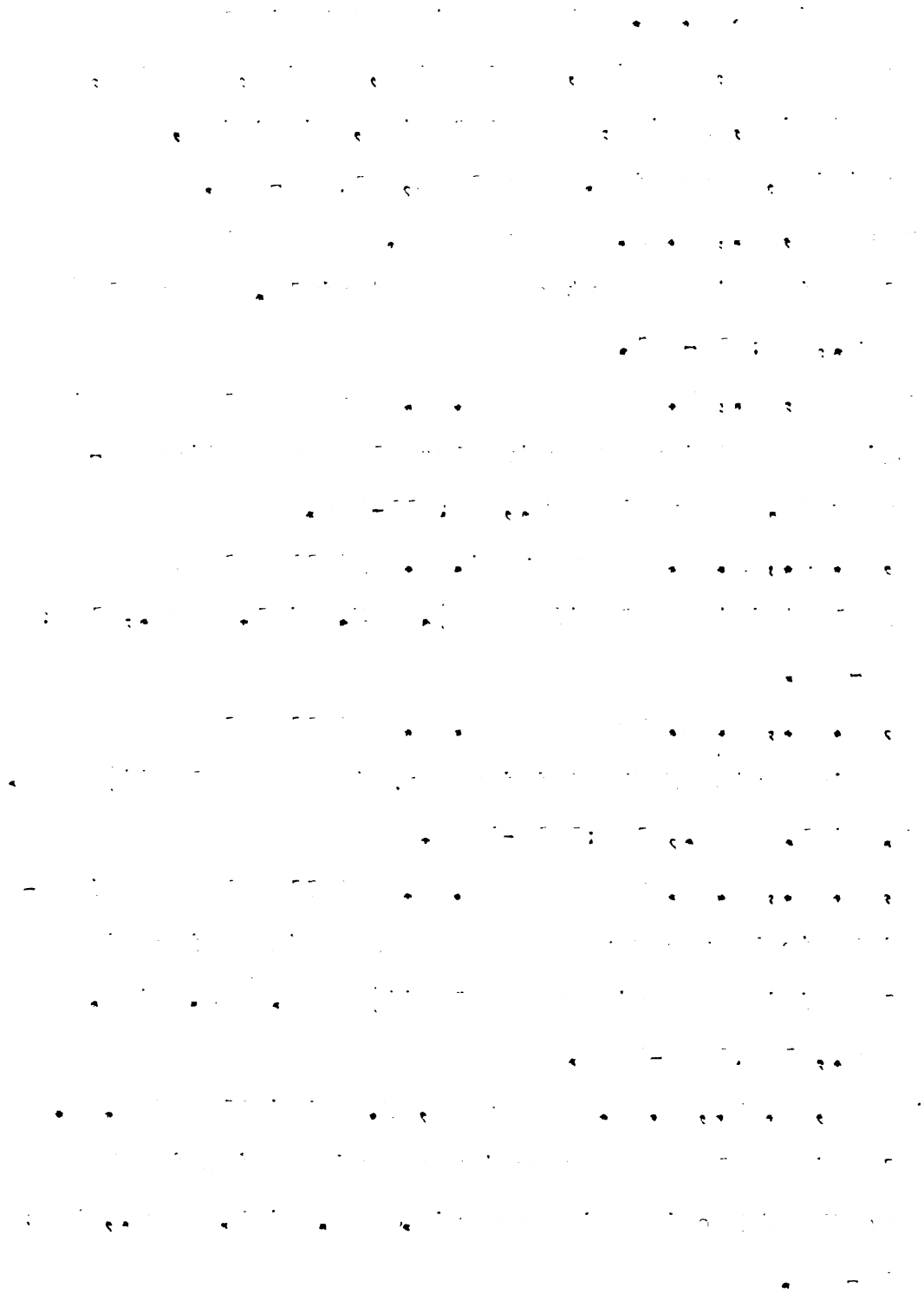

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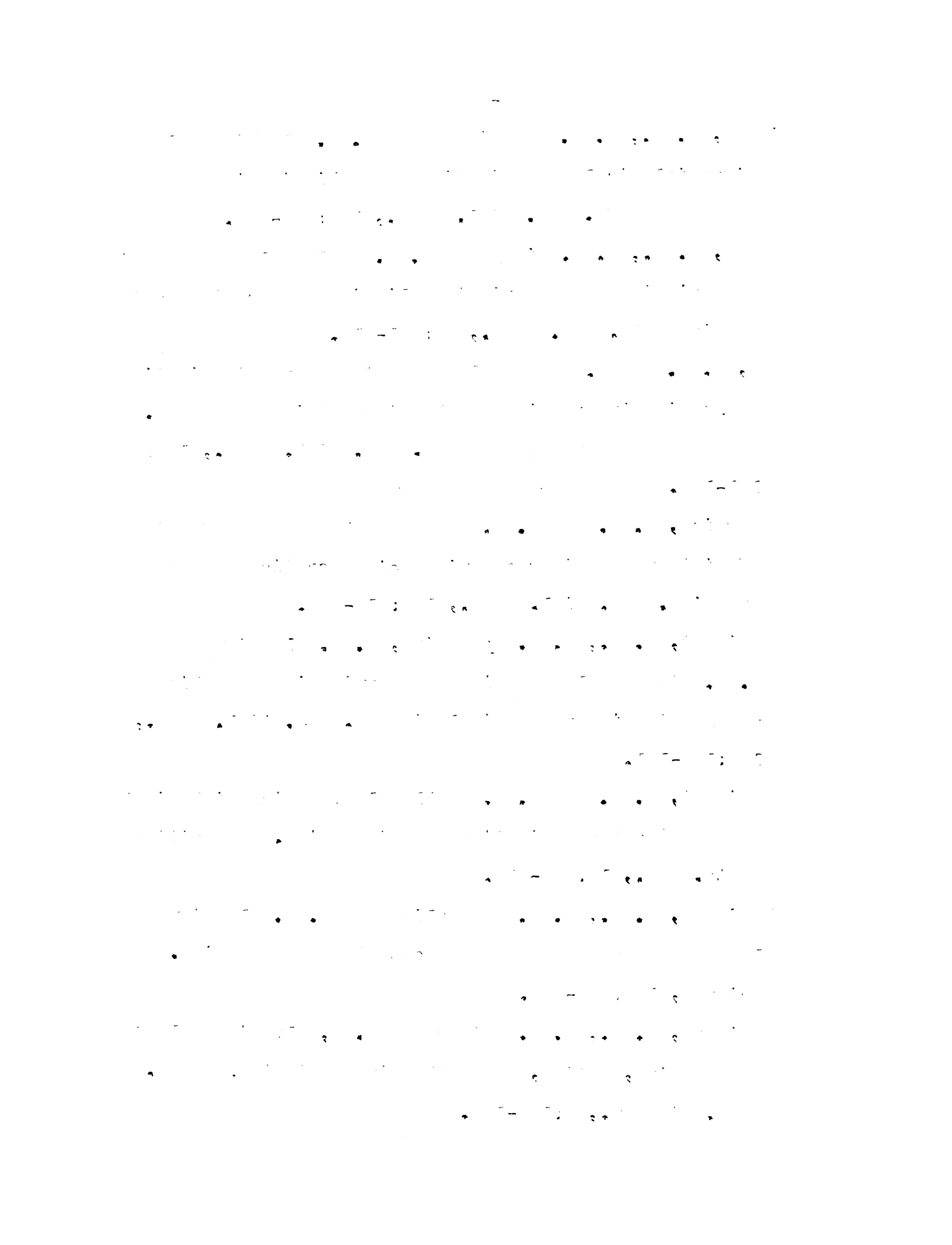

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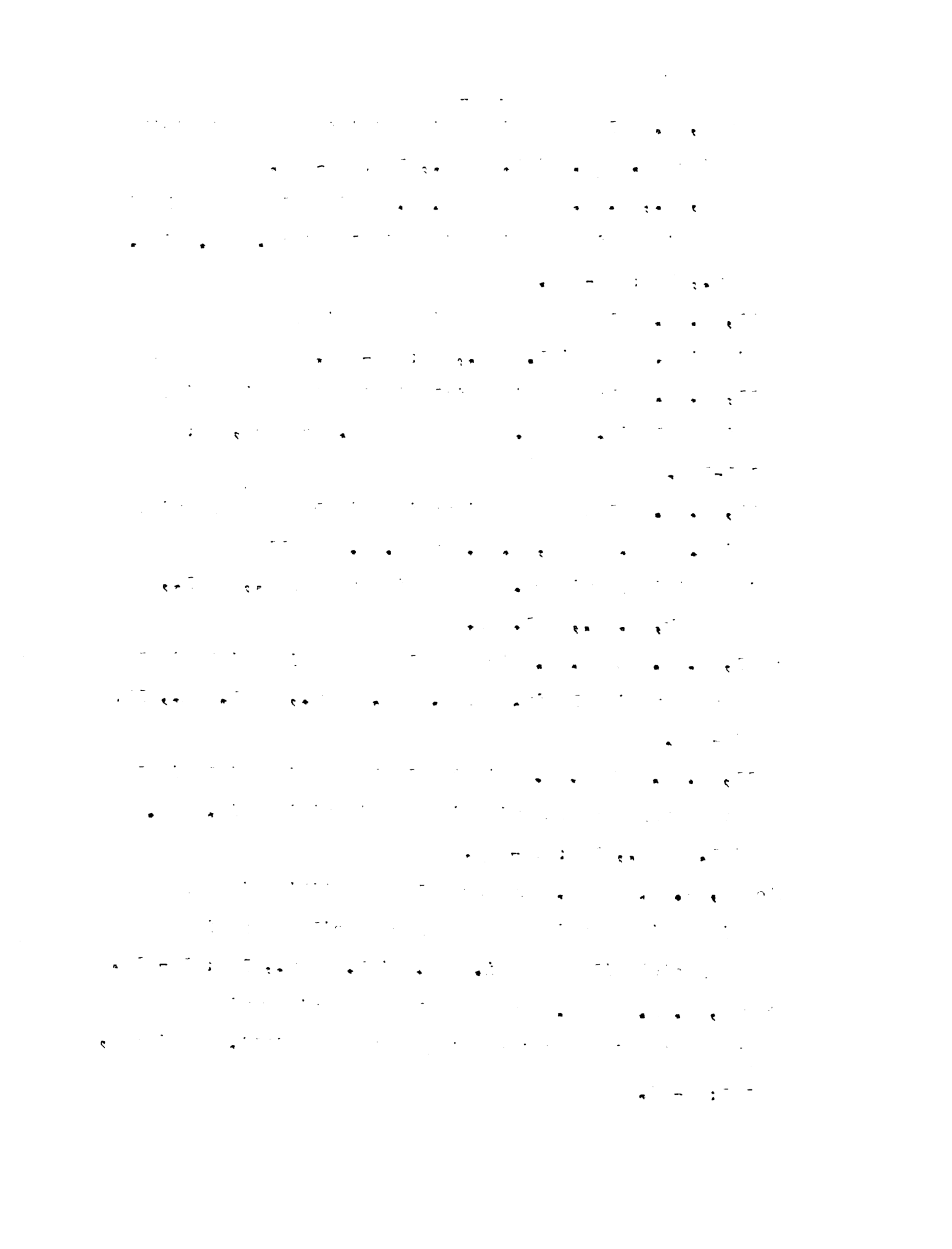

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APPENDIX

Double Strength Basal Medium for Lactobacillus arabinosus 17-5*

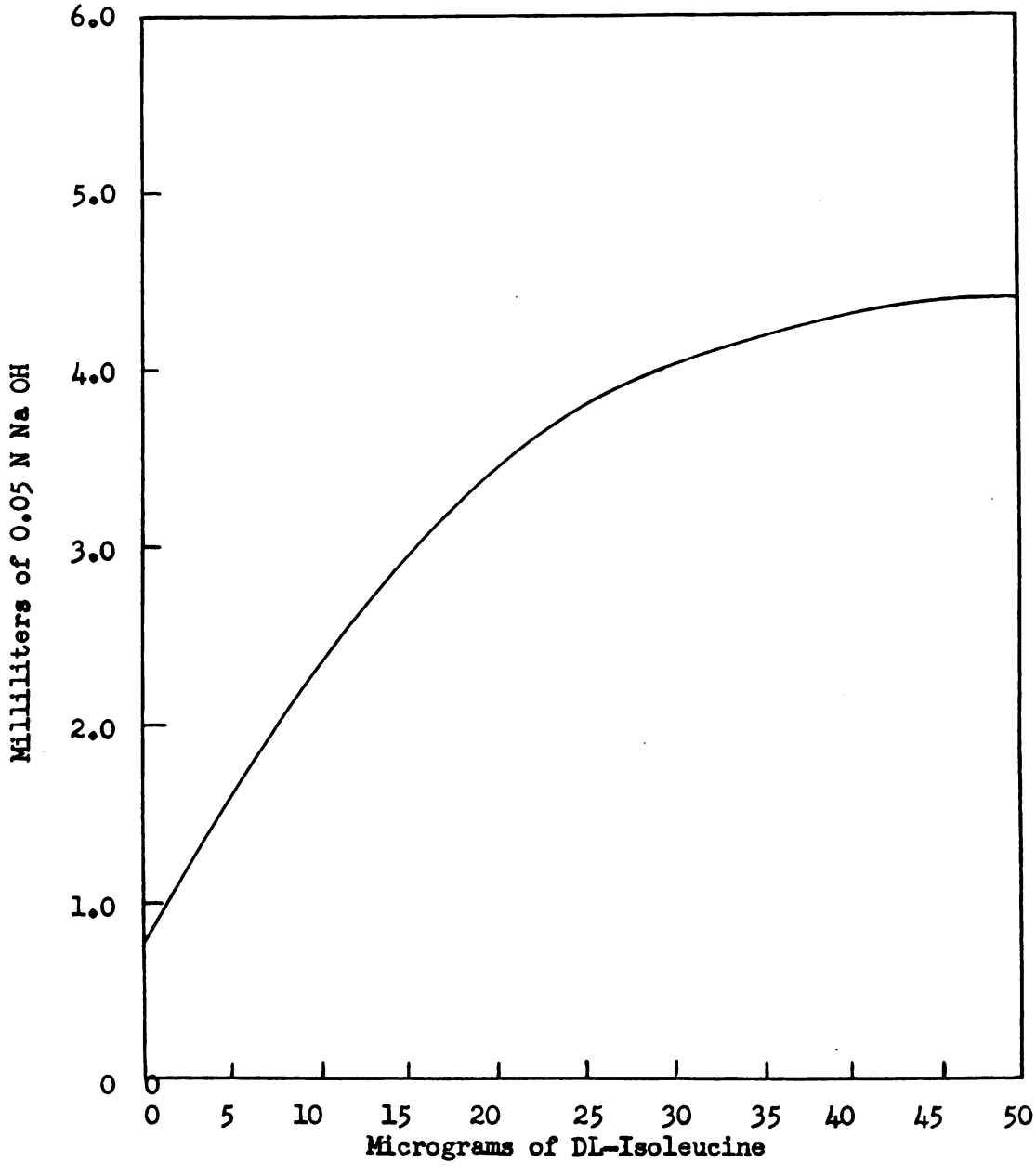
DL-Leucine	40 milligrams
DL-Isoleucine	40 "
DL-Valine	40 "
L(−)-Cystine	20 "
DL-Methionine	20 "
DL-Tryptophane	10 "
L(−)-Tyrosine	10 "
DL-Phenylalanine	20 "
L(+)-Glutamic acid	80 "
DL-Treonine	40 "
DL-Alanine	40 "
L-Asparagine	40 "
L(+)-Lysine H Cl	40 "
L(+)-Arginine H Cl	10 "
L(+)-Histidine	10 "
DL-Serine	10 "
Glycine	10 "
L(−)-Proline	10 "
Glucose	4 grams
Sodium acetate	4 "
Salts A	
$K H_2 PO_4$	100 milligrams
$K_2 H PO_4$	100 "
Salts B	
$Mg SO_4 \cdot 7H_2O$	40 "
$Fe SO_4 \cdot 7H_2O$	2 "
$Mn SO_4 \cdot 4H_2O$	2 "
Na Cl	2 "
Adenine sulfate $\cdot 2H_2O$	2 "
Guanine H Cl $\cdot 2H_2O$	2 "
Uracil	2 "
Thiamine H Cl	100 micrograms
Pyridoxine H Cl	200 "
DL-Calcium pantothenate	100 "
Riboflavin	100 "
Nicotinic acid	100 "
p-Aminobenzoic acid	20 "
Biotin	0.2 "
Folic acid	20 "
Water up to	100 milliliters

*Sauberlich and Baumann (1946).

Double Strength Basal Medium for Clostridium perfringens EP6K*

Glucose	2.0	grams
Ascorbic acid	50.0	milligrams
DL-Alanine	100.0	"
D-Arginine	50.0	"
DL-Aspartic acid	100.0	"
L-Cystine	20.0	"
Glycine	100.0	"
L-Glutamic acid	150.0	"
L-Histidine	50.0	"
Hydroxyproline	25.0	"
DL-Isoleucine	50.0	"
L-Leucine	75.0	"
L-Lysine	100.0	"
DL-Methionine	50.0	"
L-Proline	25.0	"
DL-Phenylalanine	50.0	"
DL-Serine	150.0	"
DL-Threonine	50.0	"
L-Tryptophane	50.0	"
L-Tyrosine	50.0	"
DL-Valine	75.0	"
Uracil	2.5	"
Adenine sulfate	3.4	"
Riboflavin	100.0	micrograms
Calcium D-pantothenate	200.0	"
Pyridoxamine dihydrochloride	100.0	"
Biotin	1.0	"
$MgSO_4 \cdot 7H_2O$	40.0	milligrams
$FeSO_4 \cdot 7H_2O$	2.0	"
$MnSO_4 \cdot 4H_2O$	2.0	"
NaCl	2.0	"
K_2HPO_4	1.66	grams
KH_2PO_4	0.32	"
Water up to	100	milliliters

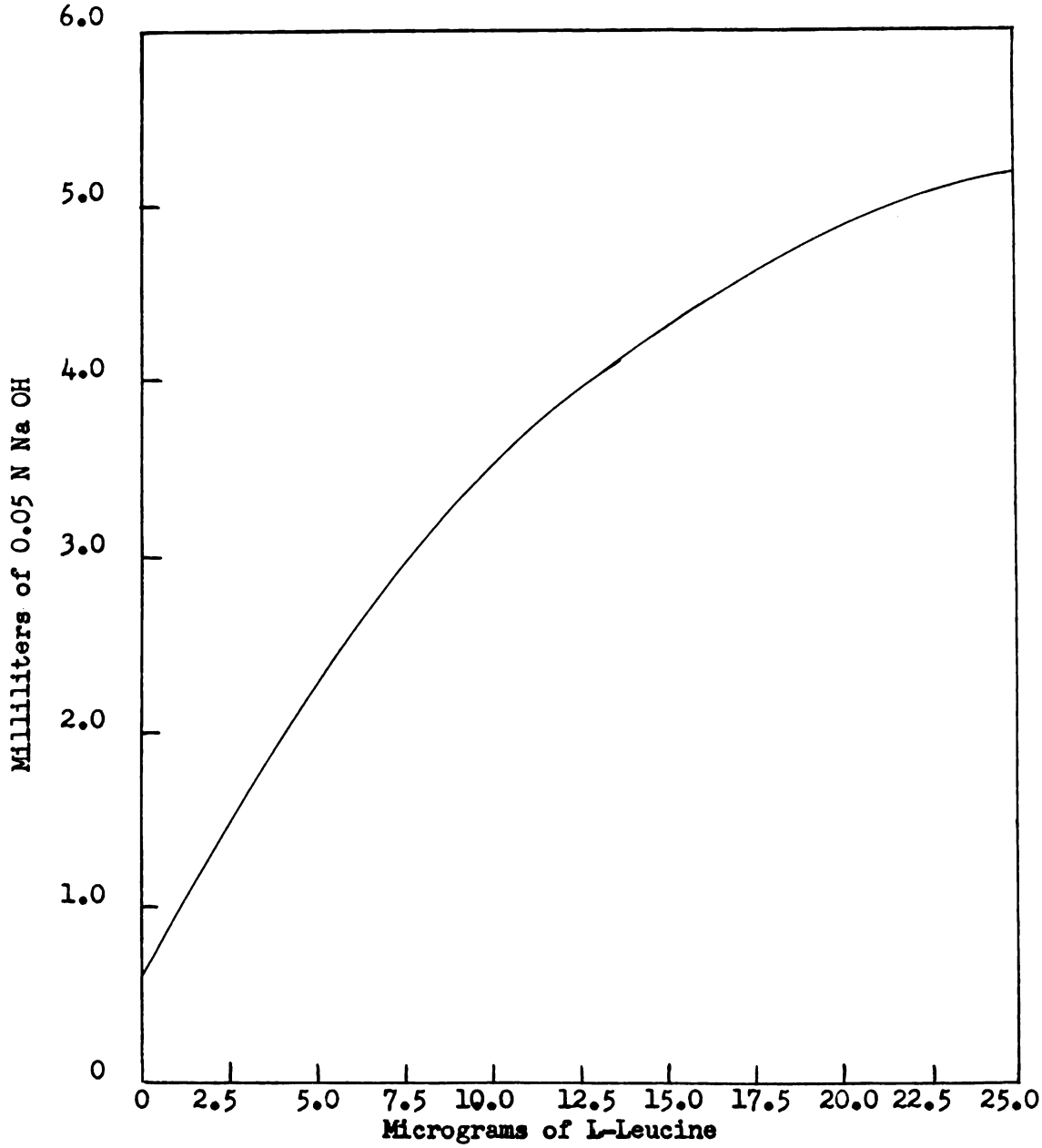
*Boyd, Logan, and Tytell (1948).



Titration Values Obtained from

Lactobacillus Arabinosus 17-5 for

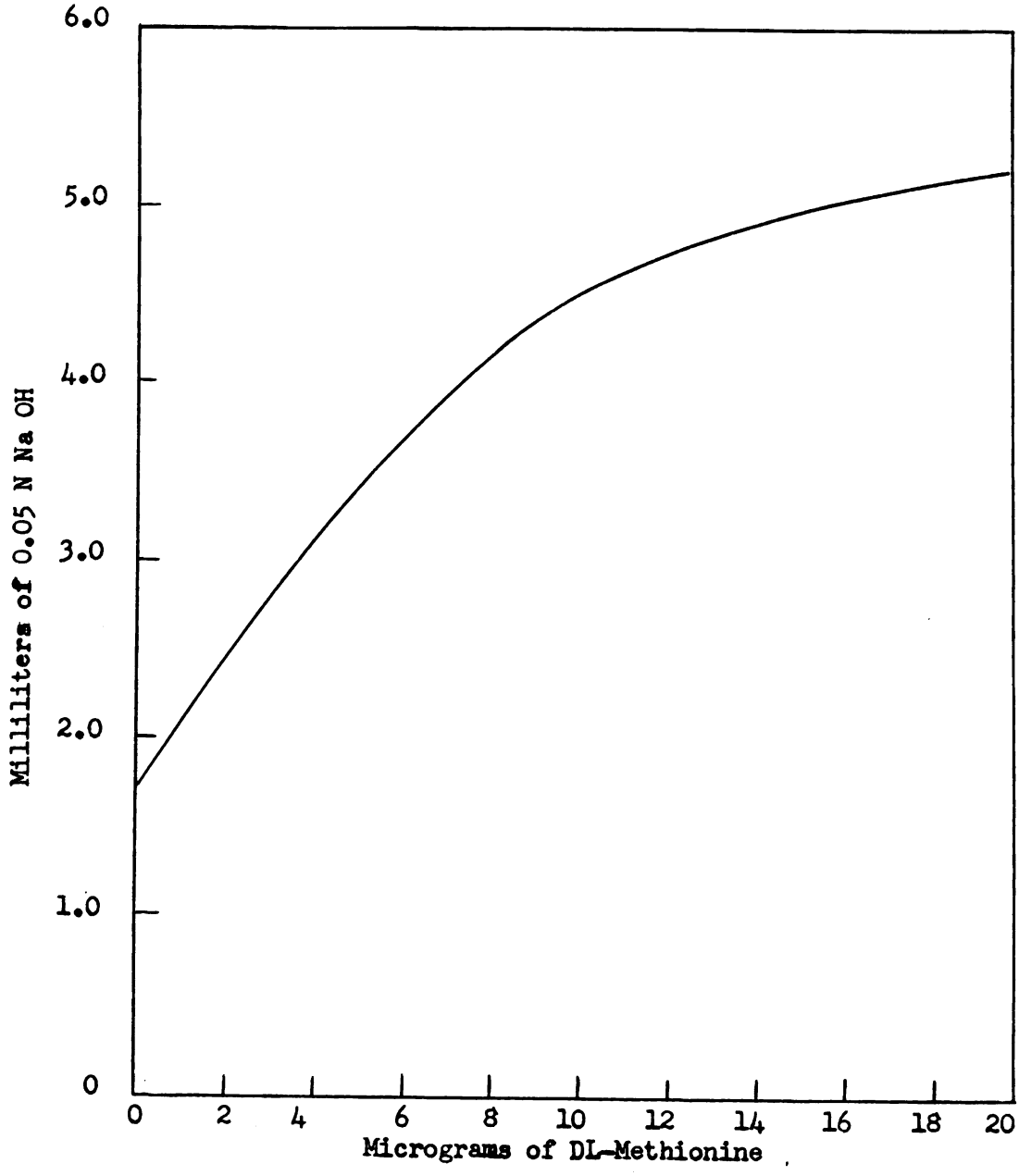
Known Concentrations of DL-Isoleucine



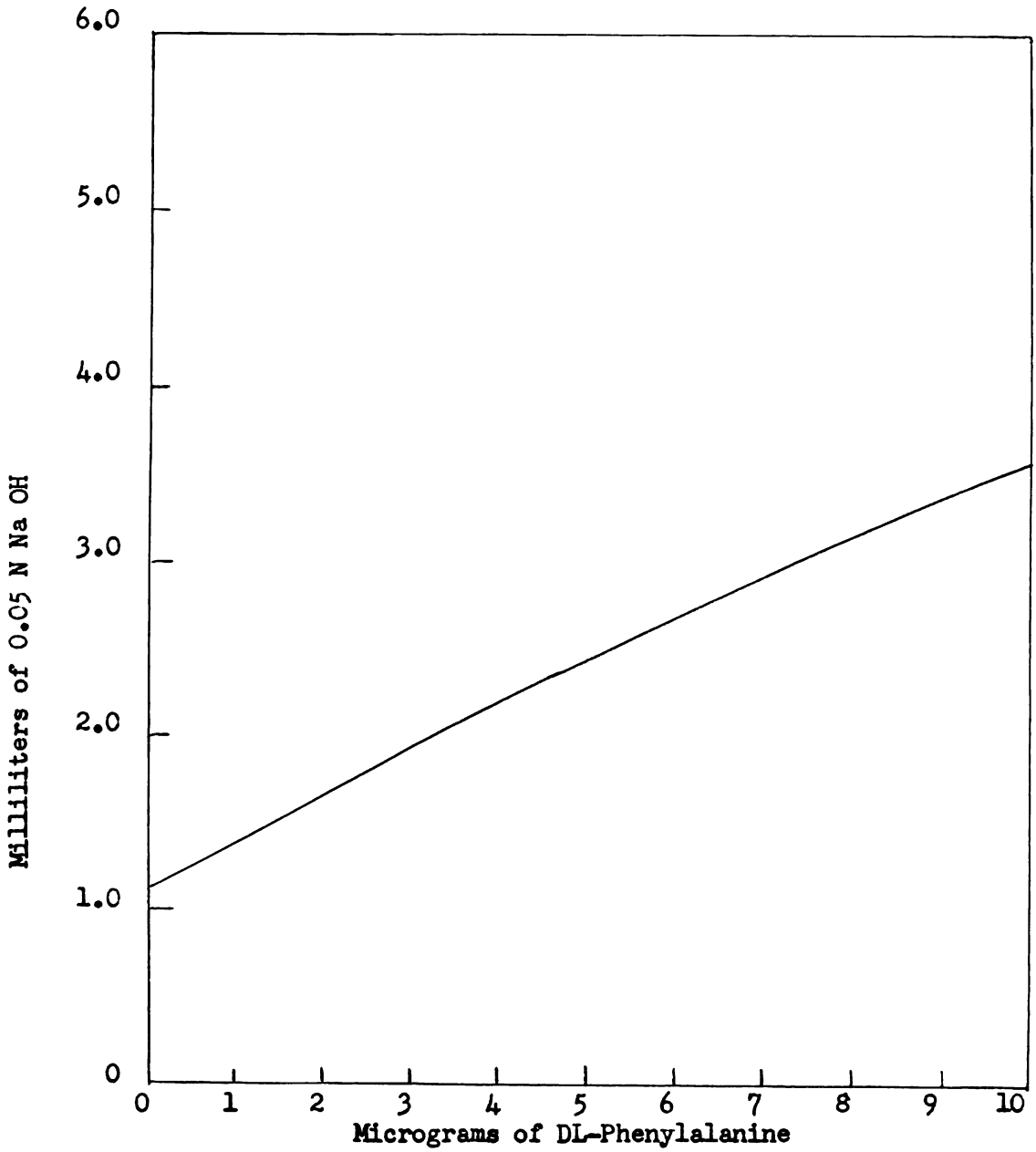
Titration Values Obtained from

Lactobacillus Arabinosus 17-5 for

Known Concentrations of L-Leucine



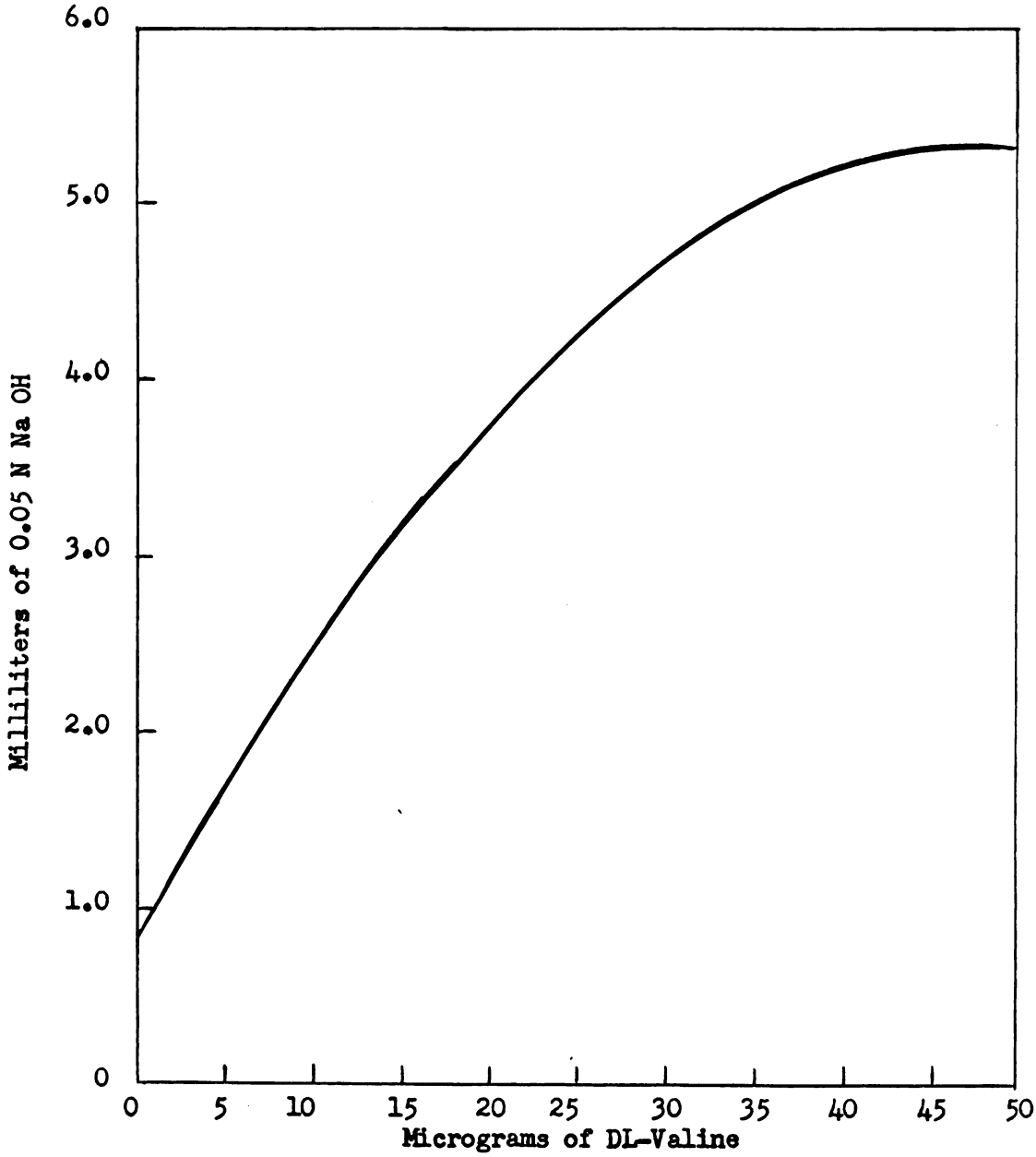
Titration Values Obtained from
Lactobacillus Arabinosus 17-5 for
Known Concentrations of DL-Methionine



Titration Values Obtained from

Lactobacillus Arabinosus 17-5 for

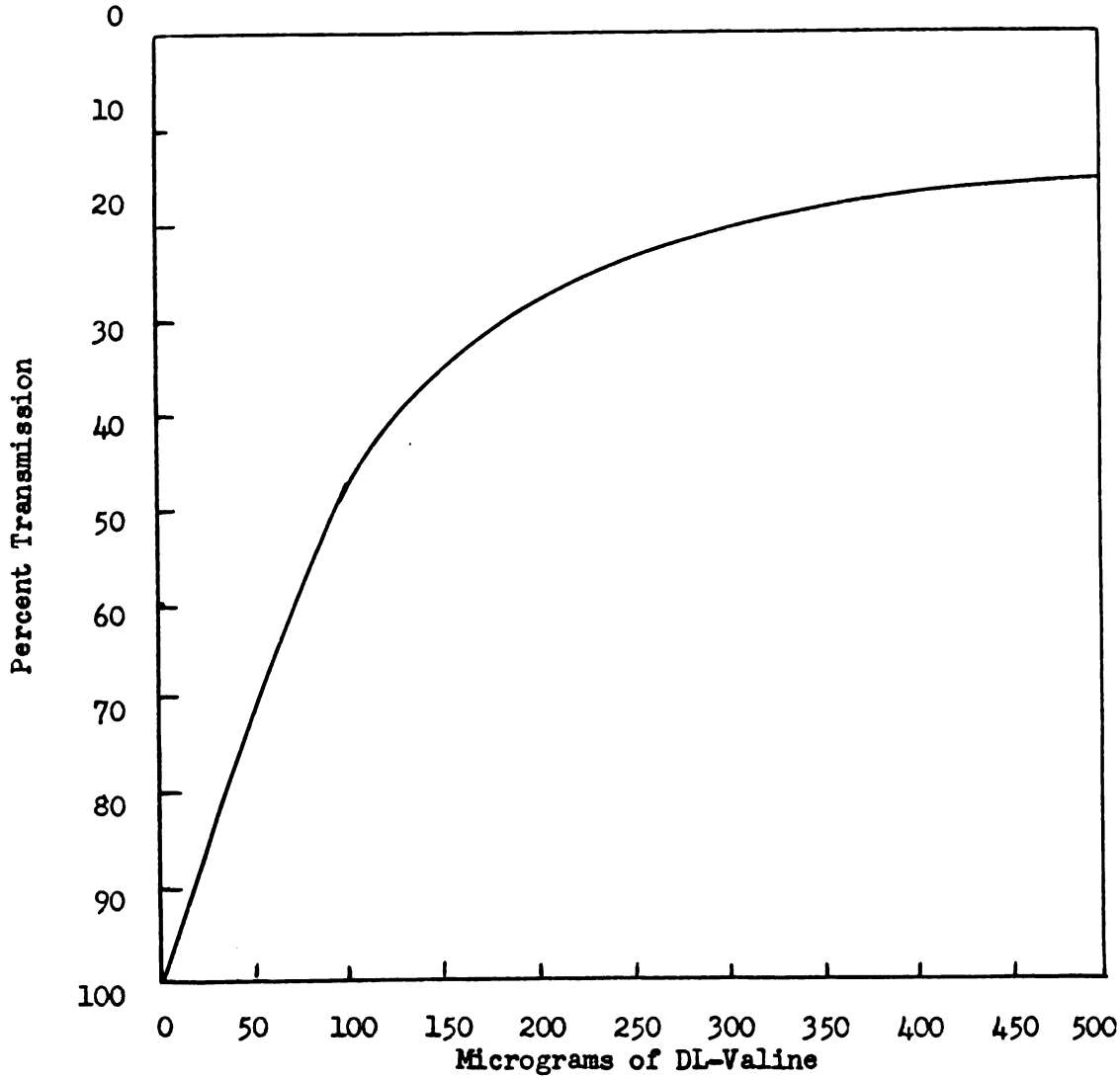
Known Concentrations of DL-Phenylalanine



Titration Values Obtained from

Lactobacillus Arabinosus 17-5 for

Known Concentrations of DL-Valine



Percent Transmissions Obtained from

Clostridium Perfringens BP6K for Known

Concentrations of DL-Valine

Selected Experimental Values for the Isoleucine Content of Casein

Casein Sample	Microbiological Method	
	L. arabinosus 17-5	C. perfringens EP6K
	percent	percent
1	5.52	5.61
	5.31	5.86
	5.36	6.12
2	5.52	5.61
	5.31	5.86
	5.36	5.94
3	5.52	5.35
	5.31	5.35
	5.36	5.35
4	5.95	5.61
	5.31	5.86
	5.36	6.12
5	5.95	5.86
	5.52	5.35
	5.36	5.35
6	5.95	5.61
	5.52	5.48
	5.48	5.48
7	5.95	5.61
	5.52	5.86
	5.61	5.86
8	5.52	5.35
	5.52	5.94
	5.61	5.94
9	5.52	5.35
	5.52	5.86
	5.61	5.86
10	5.95	6.12
	5.95	5.86
	5.52	5.86
Average	5.56	5.71

Selected Experimental Values for the Leucine Content of Casein

Casein Sample	Microbiological Method	
	L. arabinosus 17-5	C. perfringens NP6K
	percent	percent
1	11.05	10.20
	9.76	9.78
	8.42	9.78
2	9.35	10.20
	8.92	9.78
	8.67	9.78
3	10.20	10.20
	8.92	9.78
	9.95	9.78
4	11.05	10.20
	9.35	9.78
	9.95	9.78
5	9.35	10.20
	8.50	9.78
	9.75	9.78
6	10.20	10.20
	8.93	9.35
	8.67	9.35
7	10.20	10.20
	8.93	9.35
	8.92	9.35
8	9.35	10.20
	9.35	9.35
	8.93	9.35
9	10.20	9.35
	9.35	9.35
	8.93	9.35
10	10.20	9.35
	9.35	9.35
	8.92	9.35
Average	9.46	9.71

Selected Experimental Values for the Methionine Content of Casein

Casein Sample	Microbiological Method	
	L. arabinosus 17-5	C. perfringens BP6K
	percent	percent
1	3.06	2.68
	2.72	2.69
	2.65	2.69
2	3.06	2.84
	3.06	2.84
	2.75	2.84
3	2.72	2.68
	2.75	2.68
	2.86	2.68
4	2.72	2.68
	2.89	2.69
	2.75	2.69
5	2.72	2.77
	2.72	2.87
	2.96	2.87
6	2.38	2.69
	2.72	2.87
	2.86	2.69
7	2.72	2.68
	2.89	2.87
	2.65	2.87
8	2.72	2.68
	2.72	2.69
	2.89	2.69
9	3.06	2.68
	2.72	2.87
	2.96	2.69
10	3.06	2.68
	2.55	2.87
	2.86	2.87
Average	2.79	2.76

Selected Experimental Values for the Phenylalanine Content of Casein

Casein Sample	Microbiological Method	
	L. arabinosus 17-5	C. perfringens EP6K
	percent	percent
1	4.53	5.10
	5.10	5.10
	5.10	5.10
2	5.10	5.10
	4.96	5.10
	4.84	5.10
3	4.25	5.10
	4.96	4.97
	4.84	5.10
4	4.53	4.97
	5.10	4.97
	4.68	4.97
5	5.67	4.97
	4.82	4.97
	4.68	4.97
6	4.53	4.97
	4.96	5.10
	4.59	5.10
7	4.53	4.97
	4.96	4.97
	5.10	5.10
8	4.53	5.10
	4.82	4.97
	4.76	4.97
9	4.53	4.97
	4.68	5.10
	4.68	4.97
10	5.67	5.10
	4.96	4.97
	4.68	5.10
Average	4.84	5.04

Selected Experimental Values for the Valine Content of Casein

Casein Sample	Microbiological Method	
	L. arabinosus 17-5	C. perfringens NP6K
	percent	percent
1	6.38	7.39
	6.16	6.00
	6.12	6.48
2	6.38	7.39
	6.16	6.00
	6.25	6.48
3	6.38	7.14
	6.16	7.14
	6.12	7.14
4	6.38	7.39
	6.16	6.40
	6.12	6.00
5	6.38	7.39
	6.16	6.38
	6.12	6.40
6	5.95	7.39
	5.95	6.38
	6.25	6.80
7	6.38	7.39
	5.95	6.38
	5.99	6.40
8	6.38	7.14
	6.16	6.38
	6.25	6.40
9	5.95	7.39
	6.16	6.38
	5.86	6.40
10	5.95	7.14
	6.16	6.38
	6.18	6.40
Average	6.17	6.73

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