STUDIES OF THE NITROGEN METABOLISM
OF SELECTED MYCOBACTERIA

By

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INTRODUCTION

If chemical and physical changes could be detected in a microorganism as a result of its adaptation to a specific substrate under defined conditions, a knowledge of those changes conceivably would provide important clues as to the nature of the mechanisms involved in metabolizing the specific substrate. Such studies coupled with an attempt at an integration of the facts learned from the usual enzyme and metabolism studies might clarify the interrelationships among the various cellular processes, and thereby increase our understanding of the overall function of the cell as a biological unit.

It is a well established fact (1,2) that within the range of the potential enzymic constitution of an organism, wide variations in the actual enzymic constitution may occur in response to alterations in the external environment. Such variations in the enzymic constitution conceivably would be reflected in quantitative changes in the total protein of the cell as well as in the amino acid composition of that protein. Hence, the most obvious line of investigation would involve analyses of the amino acid and protein content of microorganisms after growth upon a variety of media of known composition, under defined conditions.

Numerous such investigations have been conducted in the past, the results of which have been carefully reviewed by Camien, Salle, and Dunn (3). In general, the results obtained by the earlier workers tended to support the prevalent assumption that the amino acid composition of bacterial proteins is fixed and characteristic of the organism concerned, regardless of the conditions of growth. However, the data obtained by these earlier workers could not be highly accurate because of the limitations of the analytical methods employed. With this in mind, the above authors assayed microbiologically four lactobacilli and Escherichia coli after growth upon two different media. They found no significant differences in the total nitrogen or in the quantitative content of the five to seven amino acids assayed for comparison, and concluded that their data strongly support the original hypothesis.

Freeland and Gale (4) have analyzed a number of bacteria and yeasts for amino acid content, employing the manometric, specific decarboxylase method (5). Two of the organisms studied, E. coli and Aerobacter aerogenes were grown on various buffered media, and no significant quantitative changes were noted in content of the five amino acids (arginine, lysine, histidine, tyrosine, and glutamic acid) which may be assayed for by the decarboxylase technique.

However, in the most comprehensive study of its nature to date, Stokes and Gunness (6) found evidence that while the amino acid composition of an organism is quantitatively a stable and characteristic property under fixed conditions of growth, it may vary significantly with changes in the substrate and other environmental conditions. These authors assayed for ten amino acids by microbiological procedures estimated to be accurate within 10 per cent. The data obtained for the two fungi, Streptomyces griseus and Penicillium notatum were more striking than those obtained for Bacillus subtilis. Although analyses of the latter organism showed that significantly more leucine was present in the cells after growth under one of the four environmental conditions tried, it was felt that most of the small quantitative differences in the other amino acids were within the limits of error of the analytical method.

More recently, Dunlop (7), although mainly interested in ascertaining whether E. coli synthesizes amounts of amino acids in excess of its own cellular requirements, also assayed that organism microbiologically for ten amino acids after growth upon two different media. Although he found little difference in the amino acid content of the cells grown on the two types of media, Dunlop concluded that his results varied sufficiently from those of other investigators, who employed different media, to suggest that the composition of the medium may affect the amino acid composition of the organism.

In view of the seeming contradictions and inconclusiveness of these data regarding the amino acid composition of microorganisms, particularily those relating to bacteria, it was felt that another, more exhaustive study might prove of value.

In the present investigation two closely related acid-fast bacilli of very simple nutritional requirements were studied. These bacteria have absolutely no vitamin requirements, and are able to synthesize all of their constituent amino acids from ammonium salts in the presence of a suitable carbon source and certain inorganic ions. It seemed logical that the greatest quantitative changes in the amino acid and protein content of the organisms, if such changes actually occurred, would result from variations in the source of nitrogen. Hence the organisms were grown on three media which differ, as exclusively as possible, only in the type of substrate nitrogen. After growth the organisms were assayed microbiologically for fifteen amino acids. To procure a more accurate estimate of what would constitute a "significant difference" in the analytical values obtained for any amino acid, in each instance duplicate samples were weighed, hydrolyzed, and analyzed simultaneously throughout every amino acid determination.

EXPERIMENTAL

A. Growth and Preparation of the Test Organisms

The two organisms selected for study were the aerobic, acid-fast bacilli, Mycobacterium avium* and Mycobacterium phlei*, both of which can be grown in large quantities on simple, liquid media. Growth of these aerobic bacilli on such media appears in the form of a heavy, surface pellicle, and the organisms can be harvested by ordinary filtration.

The compositions of the three media employed are given in Table I. Medium "A", in which the sole source of nitrogen is ammonium citrate, is an adaptation of the medium recommended by Long and Seibert (8) for the production of tuberculin in large quantities. The substrate nitrogen for medium "B" consists of Bacto-Peptone, a partially hydrolyzed (enzymatically) protein, and for medium "C" it consists of "vitamin free" Bacto-Casamino Acids (acid hydrolyzed casein) supplemented with tryptophane and cystine. In order to minimize the formation of precipitates in the latter two media, decreases in the amounts of phosphates and magnesium sulfate added were necessary.

After pH adjustments, the media were filtered and dispensed into florence flasks of 1 liter capacity, 500 ml to each flask. The flasks were plugged and autoclaved at 15 lbs pressure for 15 minutes. The two mycobacteria were cultured on two to six liters of each test medium. In every instance care was taken to float the inoculum on the surface of the broth.

The cultures were incubated at 37°C for three weeks. Before harvesting, the gross cultural characteristics of each organism on the three types of media were studied, and smears were prepared, stained (acid-fast)

*American Type Culture No.'s 7992 and 355, respectively.

and examined microscopically. No significant differences were observed in the cultural characteristics (macroscopic or microscopic) of either of the two organisms grown on the three different media.

The organisms were harvested by ordinary filtration, washed five to six times on the filter paper with distilled water, placed in an Arnold sterilizer for 30 minutes, quick-frozen and dried by sublimation (lyophilization). The dried bacilli were extracted (continuous Soxhlet extractors) with "peroxide free" ether for 18 - 24 hours, and then dried over anhydrous calcium chloride in a vacuum desiccator for 24 - 48 hours.

All nitrogen determinations were performed in triplicate. The 250 mg samples of the dried, defatted bacilli were weighed on an analytical balance and digested by the accepted, Kjeldahl-Gunning procedure, employing conc. H_2SO_4 in the presence of K_2SO_4 and $CuSO_4$. Upon liberation the NH_3 was collected in known aliquots of 0.2 N H_2SO_4, which were then back-titrated with 0.1 N NaOH in the presence of methyl red indicator.

B. Microbiological Assays for Amino Acids

Stock cultures of the assay organisms, <u>Lactobacillus plantarum</u> 17-5, <u>Leuconostoc mesenteroides</u> P-60, and <u>Streptococcus faecalis</u> were carried as stabs on a solid medium of the following composition per 100 ml:

Agar	1.0	gm
Peptone	0.8	
Yeast extract	0.1	
Na acetate (anhyd.)	0.1	
Glucose	1.0	
K_2HPO_4	0.05	
KH_2PO_4	0.05	
$MgSO_4 \cdot 7$ HOH	0.02	
NaCl	0.001	
$FeSO_4 \cdot 7$ HOH	0.001	
$MnSO_4 \cdot$HOH	0.001	

Fresh stock transfers were made weekly, incubated for 12 - 18 hours at 37°C and stored in the refrigerator until needed. Cultures for seeding the assay tubes were prepared from the stock stabs by transfers to tubes of the above medium with the agar omitted. These broth subcultures were incubated for 12 - 18 hours at 37°C, the cells were then centrifuged aseptically, washed once with sterile saline, and resuspended in saline for seeding the assay tubes, one drop to each tube.

The compositions of the basal media employed in the assays are given in Table II. These media were prepared from stock solutions of the various amino acids, salts, purines and pyrimidines, and vitamins. The basal media and organisms used for the various specific determinations are listed in Table III. For each determination 5 ml of the appropriate double strength basal medium, minus the amino acid in question, were added per tube, so that the final volume of each culture was 10 ml. Pyrex culture tubes, 18 x 150 mm, and glass caps were used throughout, and all sterilization was by autoclaving at 15 lbs pressure for 10 minutes.

For the standard curve determinations each level was run in triplicate. In every case graded aliquots of the pure amino acid solution were added to the standard tubes, which contained sufficient amounts of distilled water to make each final volume 5 ml before the addition of the basal medium. The L-amino acid increment in micrograms per tube of the ascending series for each standard rack is indicated in Table III.

Duplicate hydrolysates were prepared for each sample of dried, defatted organisms, and analyzed concurrently throughout every amino acid determination. The enzymatic hydrolysates for the assay of tryptophane were prepared by the method of Wooley and Sebrell (9), employing 200 mg samples and digestion with pepsin and trypsin. The acid hydrolysates used throughout the other amino acid analyses were prepared by autoclaving 1 gm samples with 6 N HCl at 15 lbs pressure for 8 hours.

From the results of preliminary assays it was possible to estimate what dilutions of the hydrolysates, and aliquots thereof, were necessary for the sample values to fall within the best range of each standard curve. In every final determination the hydrolysates were assayed in duplicate at three different levels of the standard curve. As in the preparation of the standard tubes, the final volume of each sample tube was adjusted to 5 ml with distilled water before adding the basal medium.

After inoculation and incubation at 37°C for 72 hours, the relative acid production in each assay tube was determined by transferring the contents of the tubes to beakers with distilled water and titrating to neutrality with N/10 NaOH, employing a continuous reading Beckman pH meter. Representative standard curves, one for each amino acid determination, are illustrated on the following pages.

In every assay, the final analytical value recorded for each sample of dried, defatted bacilli was calculated by averaging the results obtained for the duplicate hydrolysates. Each hydrolysate value was determined by averaging the results obtained at the three levels. The amino acid contents of the two test organisms, calculated to 16 % nitrogen, are recorded in Tables IV and V.

TABLE I.

Growth Media for the Test Organisms

Constituent	Medium (per liter) A	B	C
Di-ammonium citrate	10.0 gm	-	-
Bacto-Peptone (Difco)	-	8.0 gm	-
Casamino Acids *	-	-	18.0 gm
L-(-)-Cystine	-	-	0.05
DL-Tryptophane	-	-	0.10
Na_2CO_3 (anhyd.)	3.0	-	-
KH_2PO_4	3.0	1.0	1.0
K_2HPO_4	-	1.0	1.0
NaCl	2.0	2.0	-
$MgSO_4 \cdot 7\ HOH$	1.0	0.20	0.20
Ferric ammon. citrate	0.05	0.05	0.05
Glycerol	50.0	50.0	50.0
pH (before autoclav.)	6.9	6.6	6.9

* Difco "Vitamin Free"

TABLE II.

Media for Microbiological Assay of Amino Acids

	Medium (per 500 ml)				
Constituent	I(10)	II(11)	III(12)	IV(13)	V(14)
H_2O_2 treated peptone					7.5 gm
Casein hydrolysate				5.0 gm	
DL-α-Alanine	200 mg	100 mg	200 mg		
L(+)-Arginine•HCl	50	50	100		
L-Asparagine	200	200	200		
L(-)-Cystine	100	200	200	200 mg	100 mg
L(+)-Glutamic acid	400	400	400		
Glycine	20	20	100		
L(+)-Histidine•HCl•HOH	50	50	100		
DL-Isoleucine	200	200	200		
DL-Leucine	200	200	200		
L(+)-Lysine•HCl•HOH	200	200	200		
DL-Methionine	100	100	200		100
DL-Phenylalanine	100	100	100		
L(-)-Proline	50	50	50		
DL-Serine	50	50	200		
DL-Threonine	200	200	200		
DL-Tryptophane	50	100	100		100
L(-)-Tyrosine	50	100	100		100
DL-Valine	200	200	200		
Glucose	20 gm	20 gm	20 gm	20 gm	20 gm
Na Acetate (anhyd.)	20		20	20	12

NOTE: ( ) after Roman numerals indicate references

TABLE II. (concluded)

Constituent	Medium (per 500 ml)				
	I	II	III	IV	V
Na Citrate·HOH		25 gm			
NH_4Cl					6 gm
KH_2PO_4	500 mg		500 mg	500 mg	500 mg
K_2HPO_4	500	5	500	500	500
$MgSO_4$·7 HOH	200	200 mg	200	200	200
$FeSO_4$·7 HOH	10	10	10	10	10
$MnSO_4$·4 HOH	10	10	10	10	10
NaCl	10	10	10	10	10
Adenine sulfate·2 HOH	10	10	10	10	10
Guanine·HCl·2 HOH	10	10	10	10	10
Uracil	10	10	10	10	10
Xanthine	10	10	10		
Thiamine·HCl	0.50	0.50	0.50	0.10	1.0
Pyridoxine·HCl	1.0	1.0	1.0	0.10	2.0
DL-Ca Pantothenate	0.50	0.50	0.50	0.10	2.0
Riboflavin	0.50	0.50	0.50	2.0	2.0
Nicotinic acid	1.0	1.0	1.0	0.40	2.0
PABA	0.10	0.10	0.10	0.10	0.01
Biotin	0.001	0.001	0.001	0.2 μg	0.005
Folic acid	0.01	0.01	0.01		0.0015
pH (before autoclav.)	6.6-6.8	6.6-6.8	6.6-6.8	6.6-6.8	6.9-7.0

TABLE III.

Organisms and Media Used for Determining Specific Amino Acids

Amino Acid	Organism	Std. Curve Increment µg/tube	Medium	Range of Disagreement* Dupl. Samples
Glutamic acid	*L. plantarum*	10	I **	0.6 - 2.5
Leucine	"	5	I	0.3 - 1.4
Isoleucine	"	5	I ***	0.3 - 3.5
Valine	"	5	I ***	0.3 - 1.9
Phenylalanine	"	5	I	0.8 - 2.0
Threonine	*S. faecalis*	5	II	0.8 - 3.3
Arginine	"	10	II	0.1 - 0.7

* Per cent of the mean value

** + 5 mg NH_4Cl and 20 µg glutamine per tube (15,16)

*** + 20 ml tomato eluate (17) per 500 ml medium

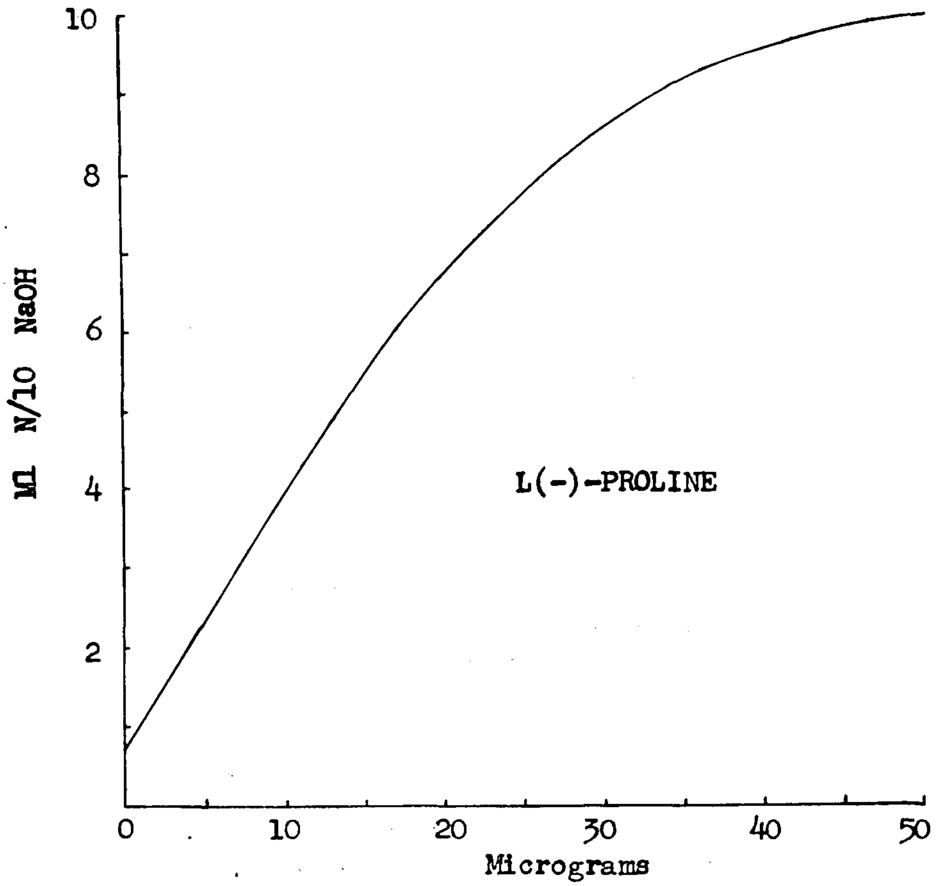
TABLE III. (concluded)

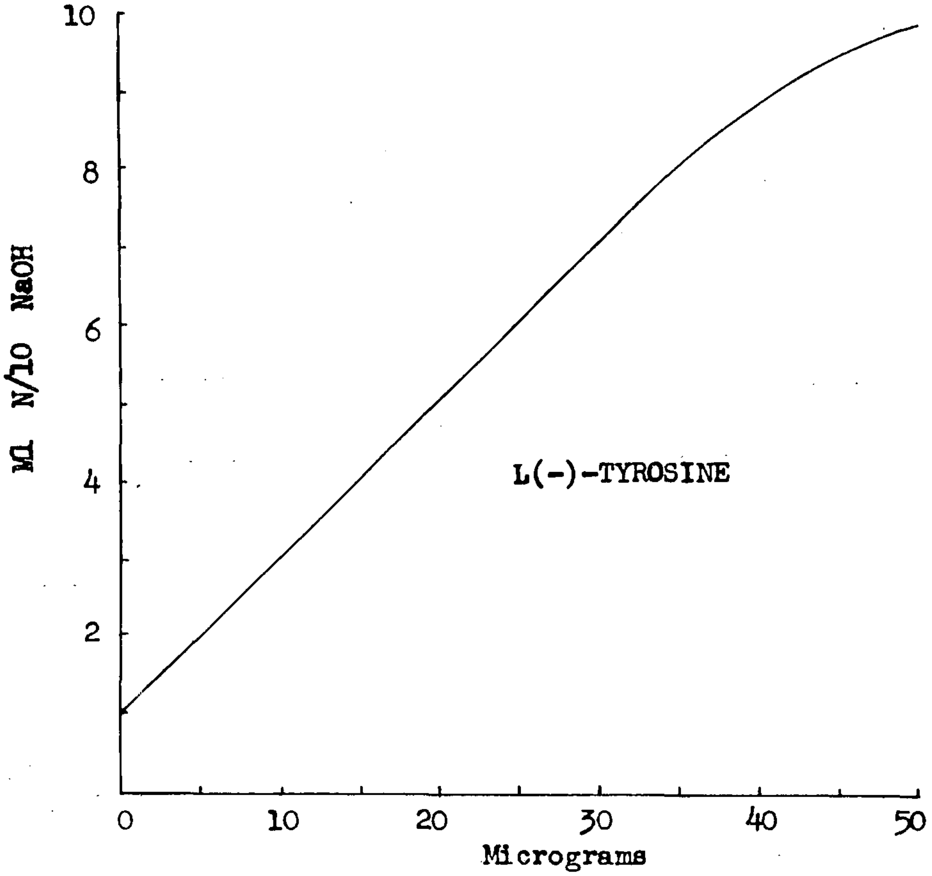
Organisms and Media Used for Determining Specific Amino Acids

Amino Acid	Organism	Std. Curve Increment µg/tube	Medium	Range of Disagreement* Dupl. Samples
Histidine	*S. faecalis*	5	II	0.0 - 1.8
Aspartic acid	*L. mesenteroides*	10	III, phosphates incr. 4x's	0.0 - 3.8
Lysine	"	25	III	0.0 - 2.7
Proline	"	5	III, + 10 µg Proline/tube	0.0 - 3.5
Tyrosine	"	5	III	0.0 - 2.6
Tryptophane	*L. plantarum*	1	IV	2.2 - 8.3
Methionine	*L. mesenteroides*	5	V	0.0 - 2.3
Cystine	"	1	V	0.0 - 6.0

* Per cent of the mean value

Representative Standard Curves





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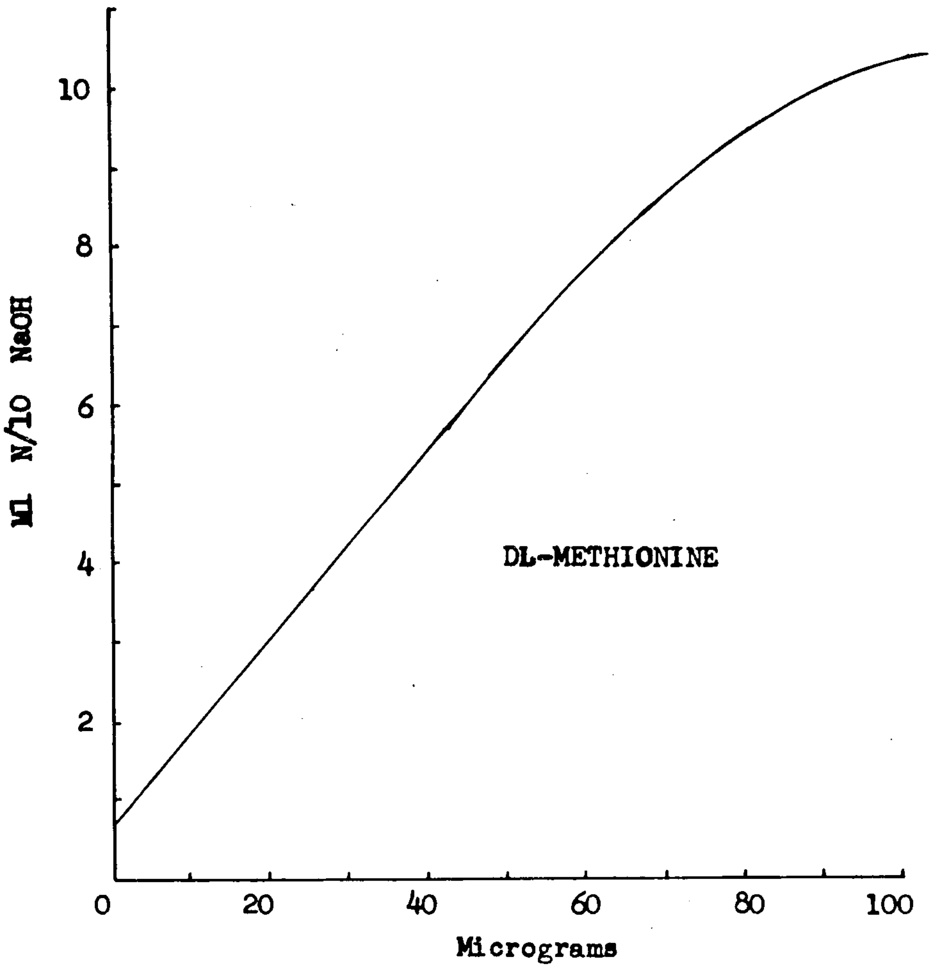

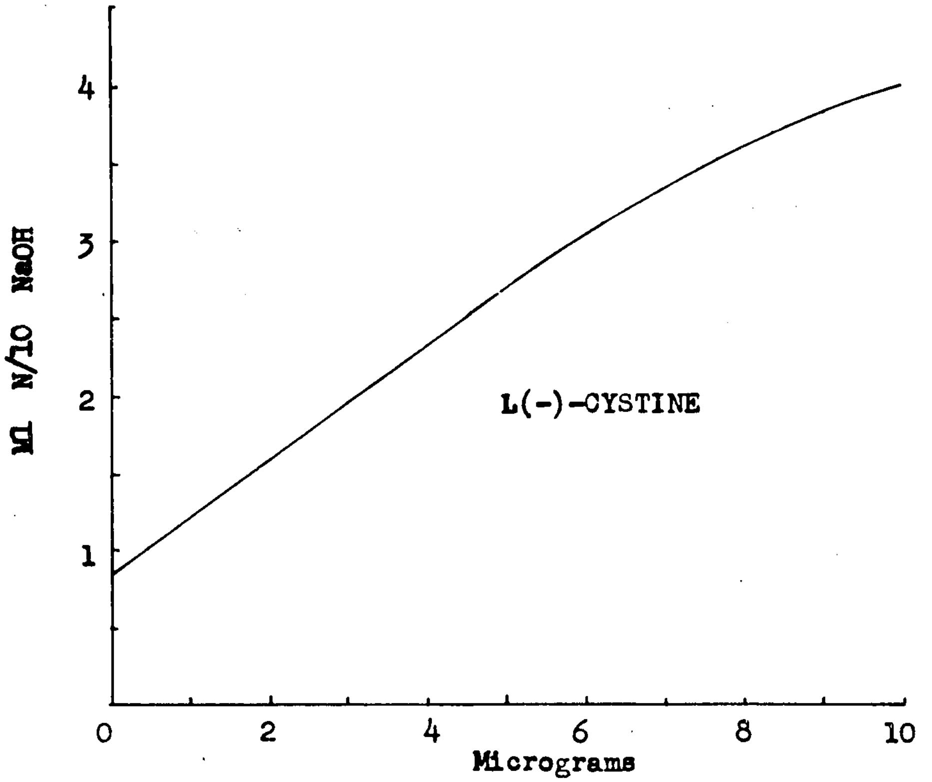

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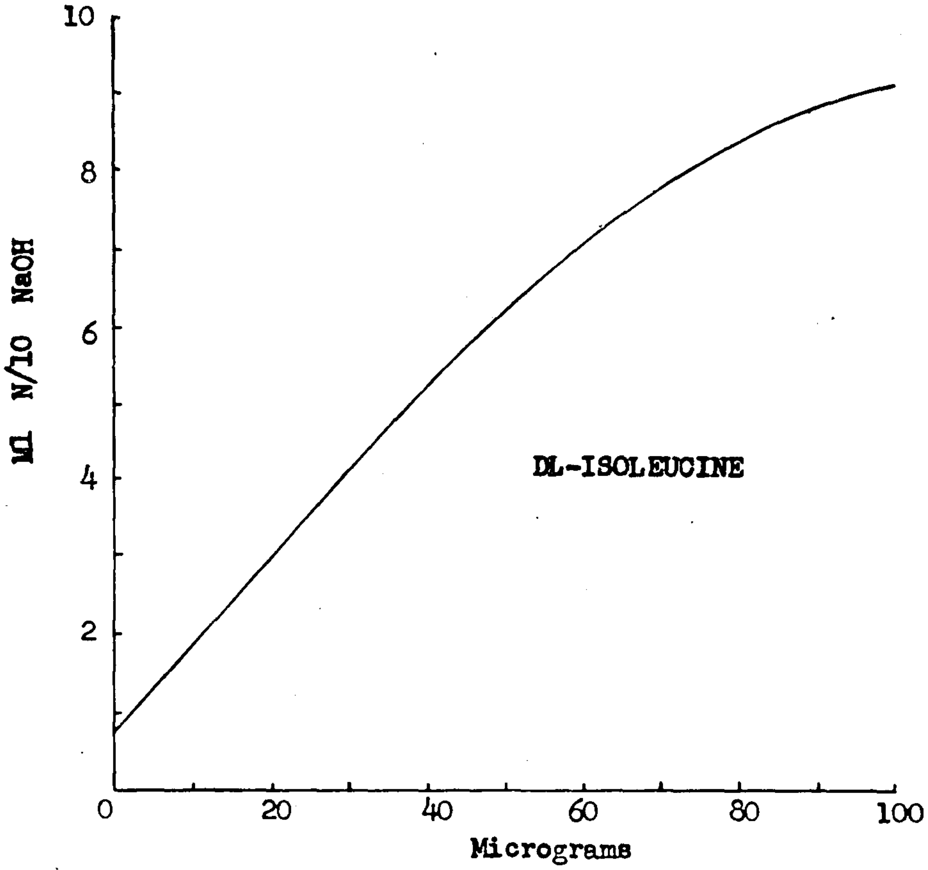

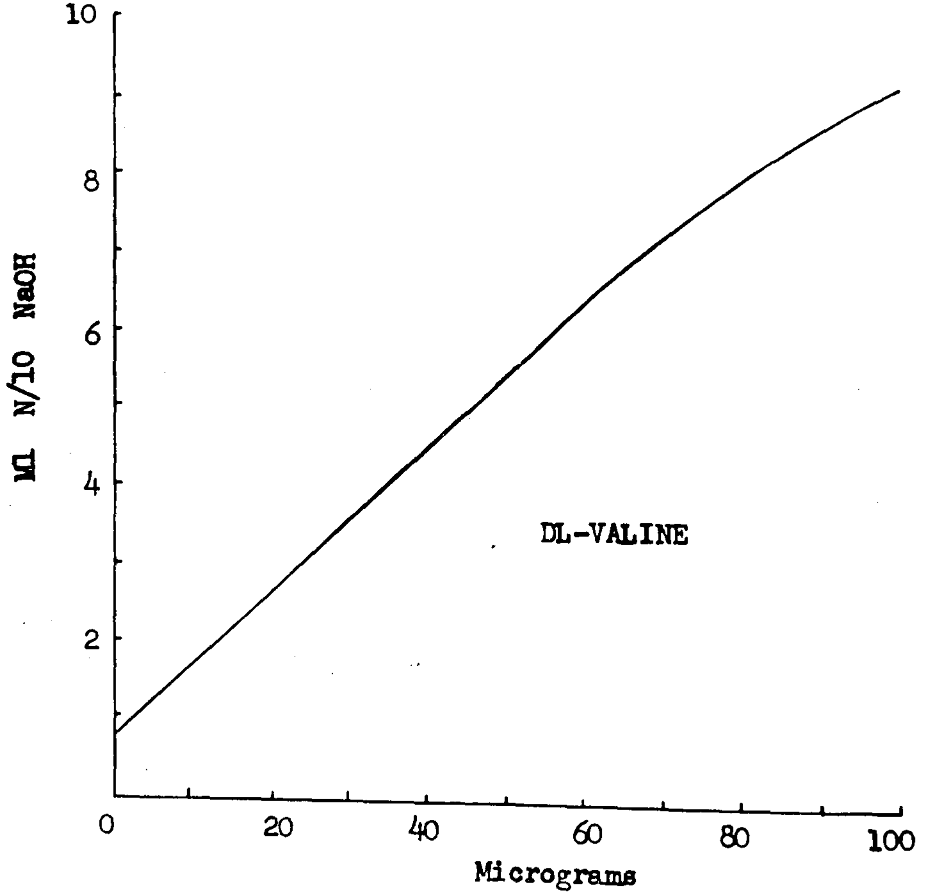

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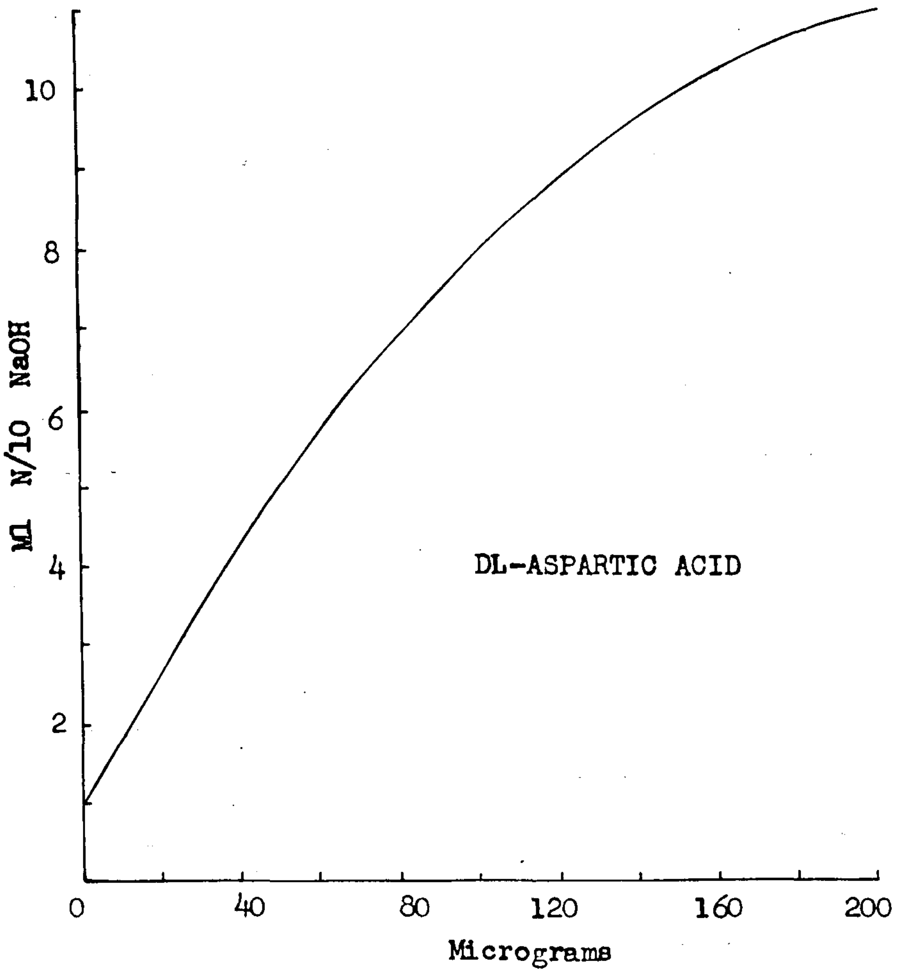

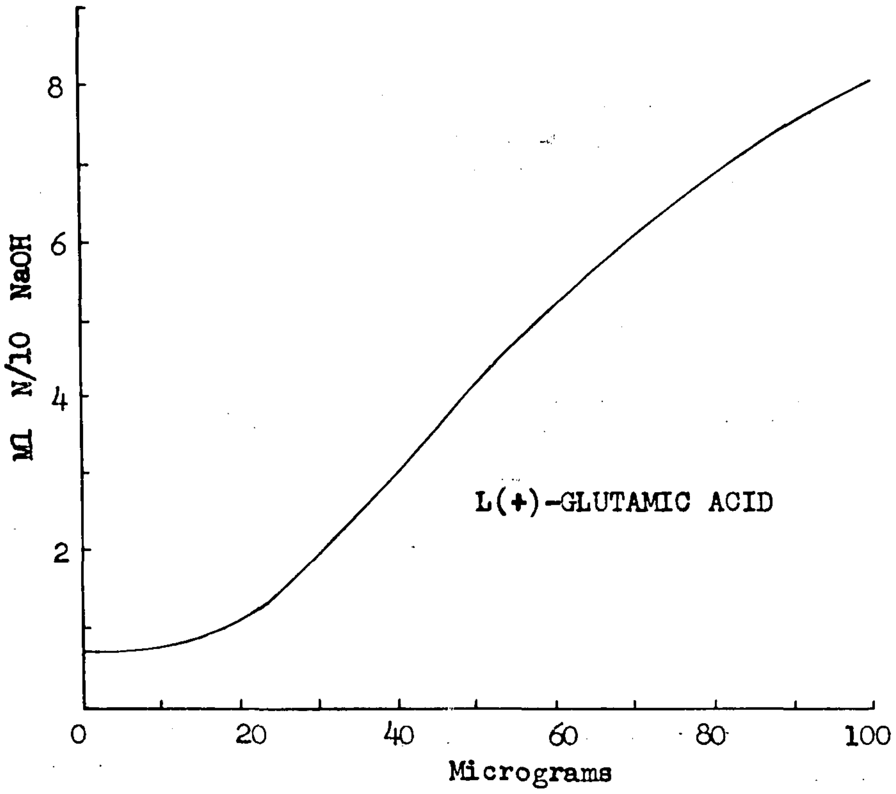

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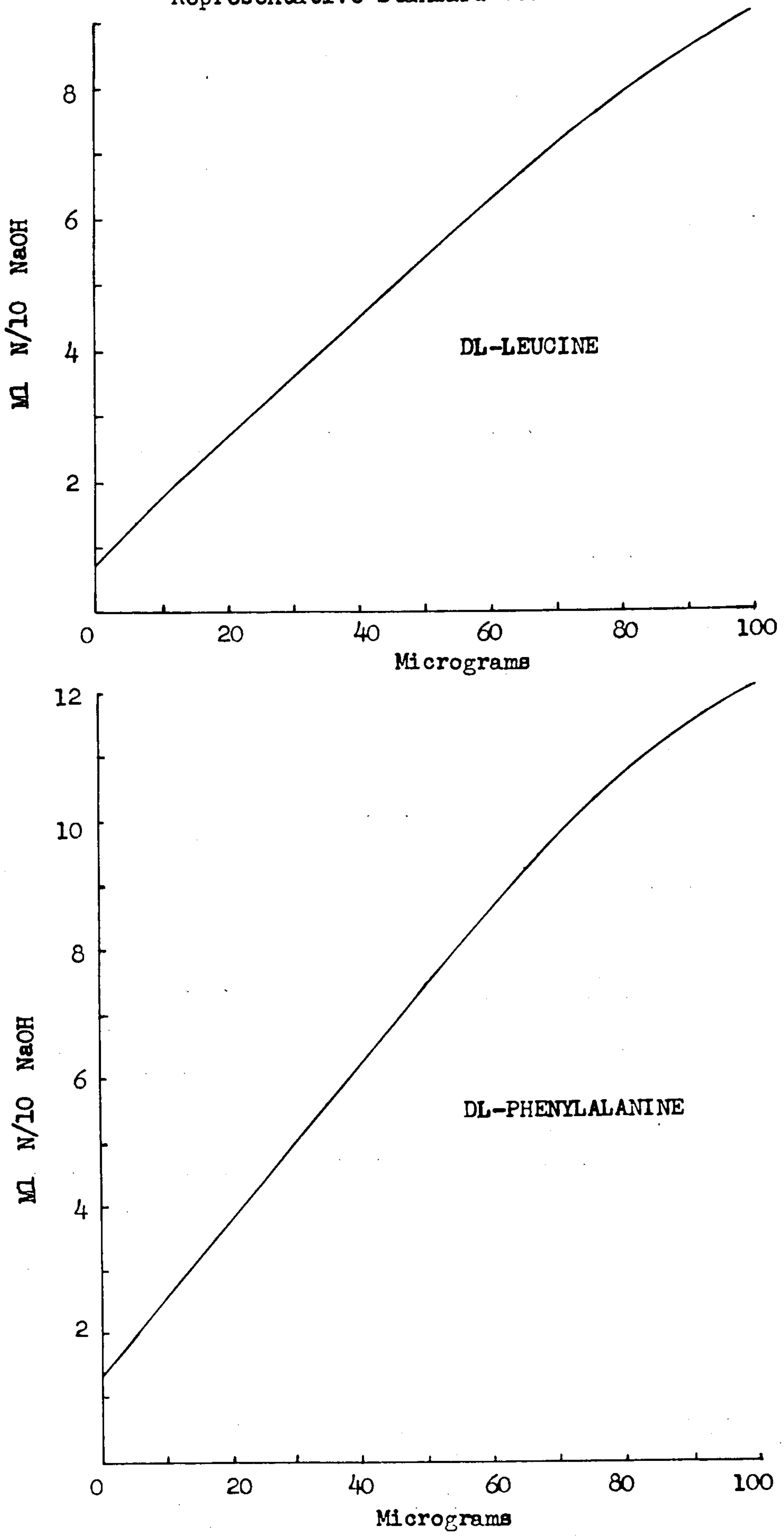

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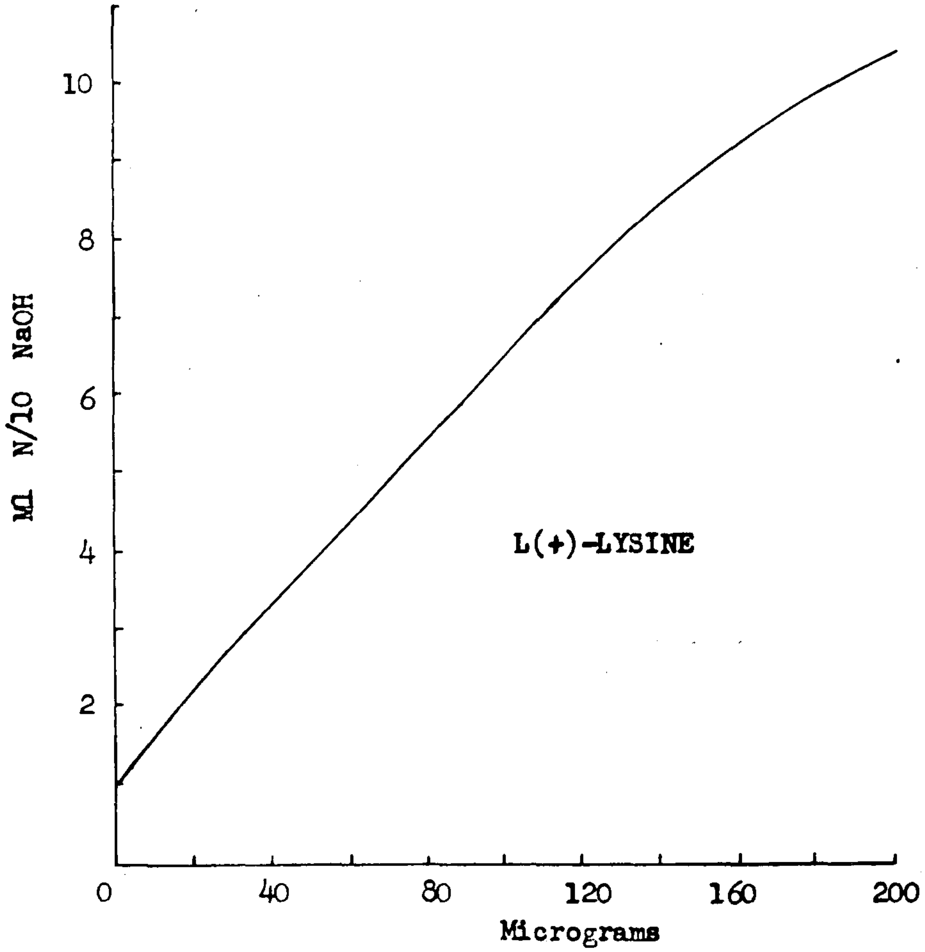

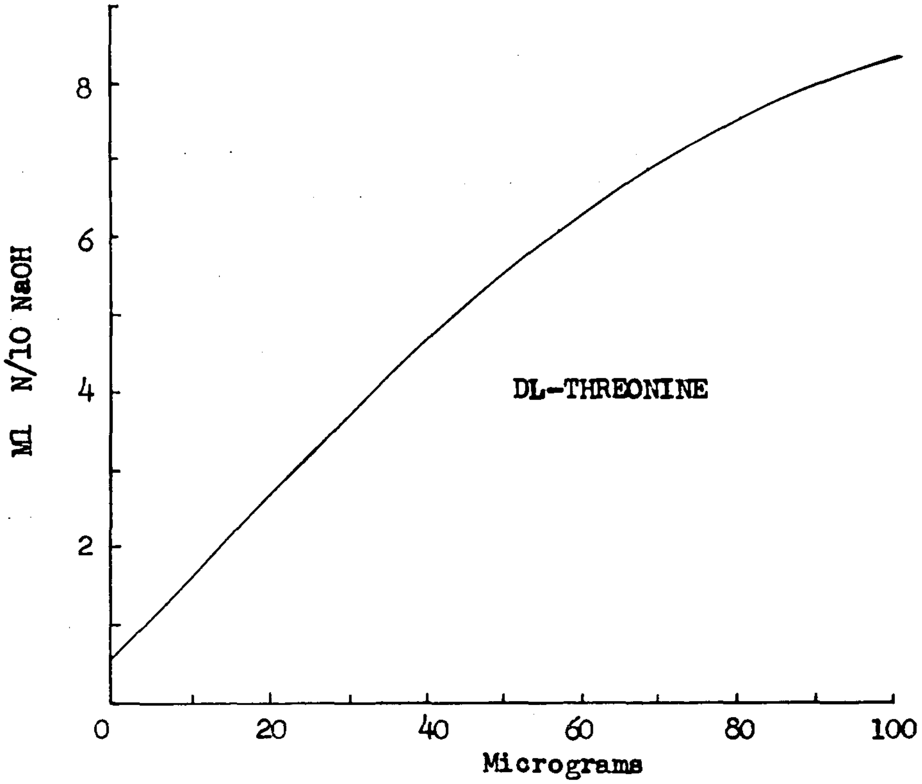

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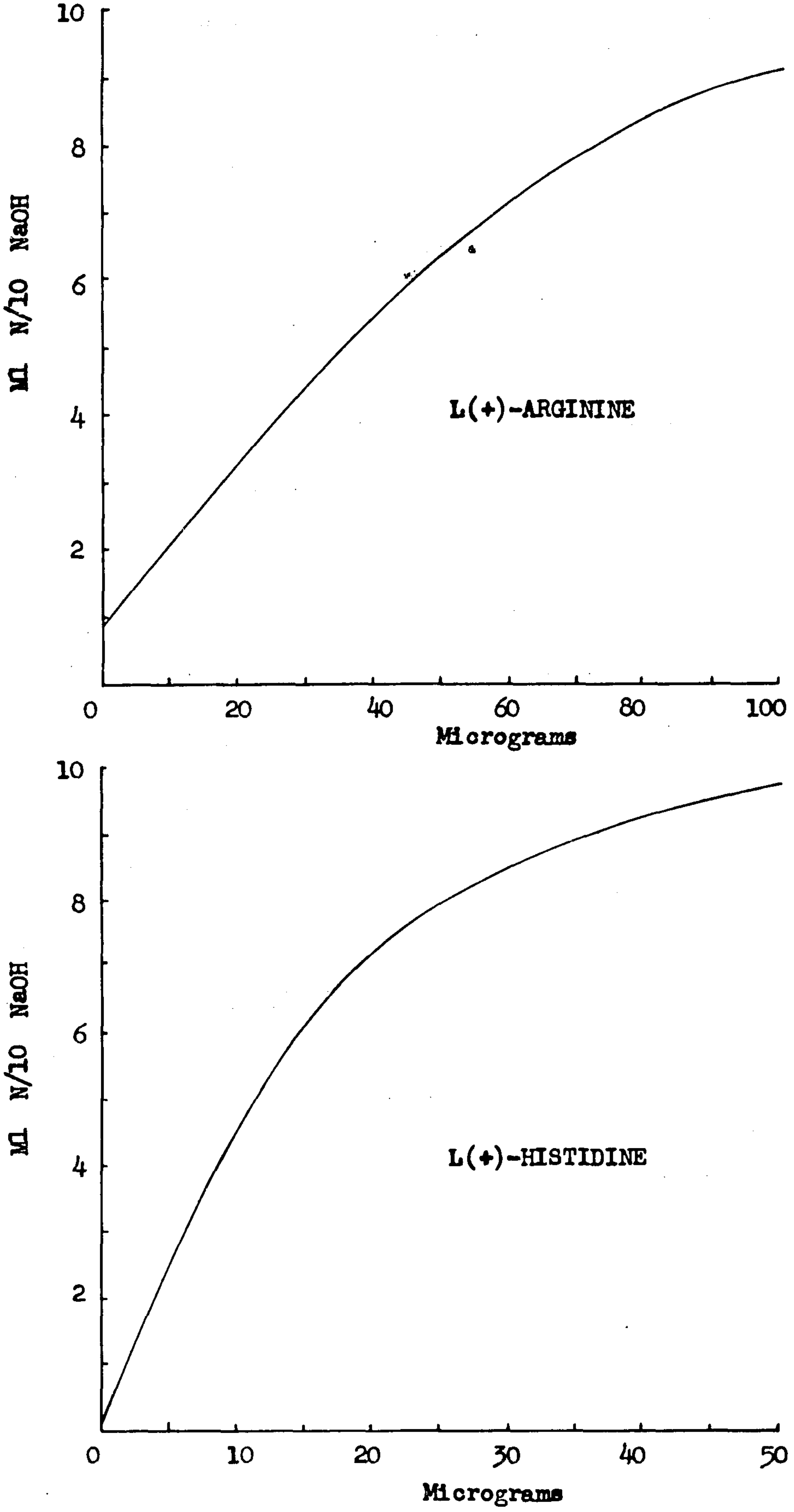

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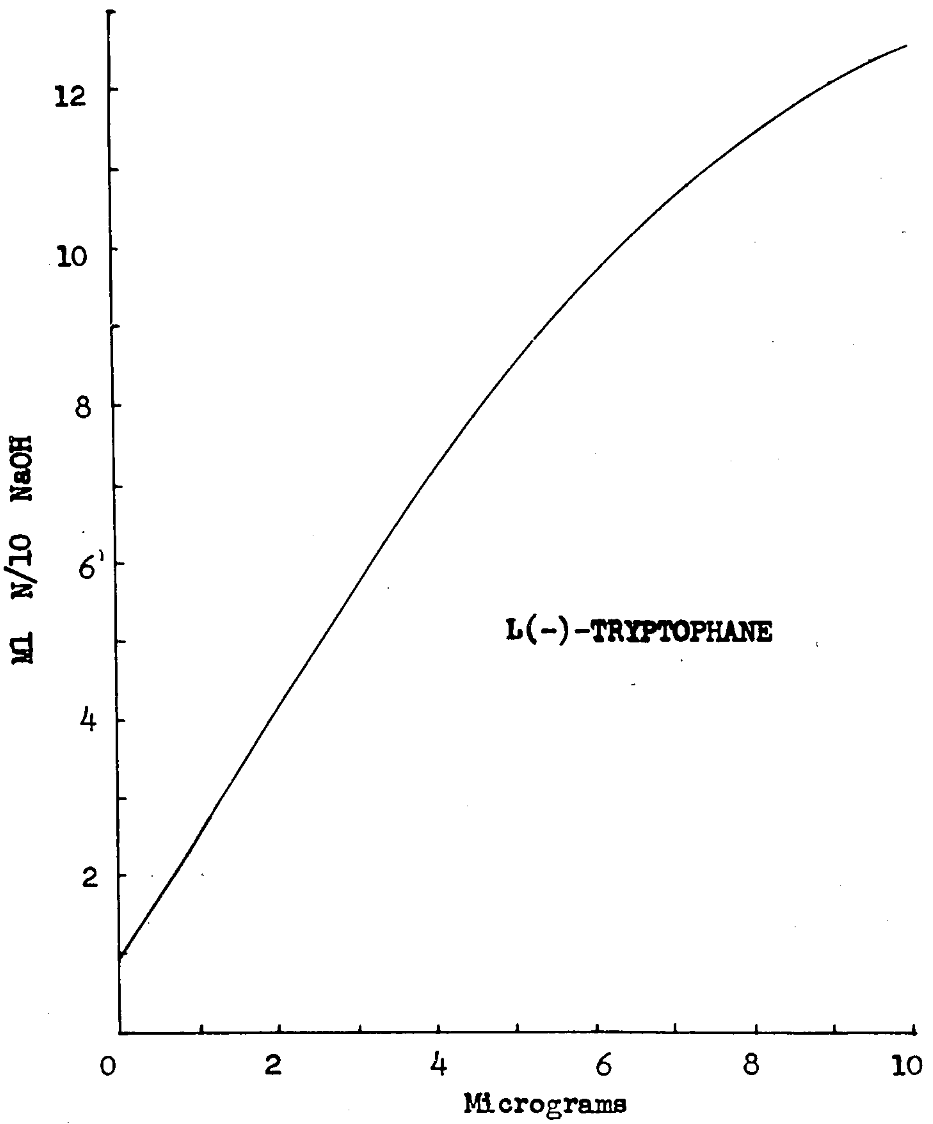


TABLE IV.

Amino Acid Content of Mycobacterium phlei

(calculated to 16 % nitrogen)

	Substrate Nitrogen		
Constituent	Ammonium Citrate	Peptone	Casamino Acids
Total nitrogen	11.36 %	10.64 %	8.65 %
Protein (N x 6.25)	71.00	66.50	54.06
Arginine	7.42	6.83	5.78
Aspartic acid	4.92	3.78	4.39
Cystine	0.251	0.221	0.251
Glutamic acid	10.62	12.34	9.81
Histidine	1.67	1.72	1.52
Isoleucine	4.55	5.81	3.98
Leucine	7.71	6.59	6.34
Lysine	3.50	3.66	2.48
Methionine	1.47	1.27	1.49
Phenylalanine	3.58	3.39	2.97
Proline	4.80	4.08	4.42
Threonine	4.96	4.36	4.68
Tryptophane	0.387	0.145	0.158
Tyrosine	2.54	1.86	1.88
Valine	6.95	5.80	5.86
Total *	65.328	61.856	56.009

* Per cent of Total Protein accounted for.

TABLE V.

Amino Acid Content of _Mycobacterium avium_

(calculated to 16 % nitrogen)

	Substrate Nitrogen		
Constituent	Ammonium Citrate	Peptone	Casamino Acids
Total nitrogen	11.55 %	9.35 %	11.34 %
Protein (N x 6.25)	72.39	58.44	70.88
Arginine	7.62	6.82	7.09
Aspartic acid	4.67	4.04	5.03
Cystine	0.213	0.248	0.175
Glutamic acid	10.25	9.11	9.43
Histidine	1.95	1.80	1.98
Isoleucine	4.48	6.85	4.53
Leucine	7.36	7.01	7.08
Lysine	3.95	4.84	2.97
Methionine	1.37	1.40	1.69
Phenylalanine	3.46	3.43	3.48
Proline	4.75	4.26	4.87
Threonine	4.98	4.84	5.23
Tryptophane	0.297	0.465	0.556
Tyrosine	2.24	1.86	2.30
Valine	6.83	6.43	7.05
Total *	64.420	63.403	63.461

* Per cent of Total Protein accounted for.

TABLE VI.

Variation* in Amino Acid Content of Bacterial Proteins With Substrate Nitrogen

Amino Acid	Bacto-Peptone		Casamino Acids	
	M. phlei	M. avium	M. phlei	M. avium
Arginine	- 7.95	-10.5	-22.1	- 6.96
Aspartic acid	-23.2	-13.5	-10.8	+ 7.70
Cystine	-11.9	+16.4	0.0	-17.8
Glutamic acid	+16.2	-11.1	- 7.63	- 8.00
Histidine	+ 2.95	- 7.70	- 8.98	+ 1.54
Isoleucine	+27.7	+53.0	-12.5	+ 1.12
Leucine	-14.5	- 4.76	-17.8	- 3.81
Lysine	+ 4.57	+22.5	-22.7	-24.8
Methionine	-13.6	+ 2.19	+ 1.36	+23.4
Phenylalanine	- 5.31	- 0.87	-17.0	+ 0.58
Proline	-15.0	-10.3	- 7.92	+ 2.53
Threonine	-12.1	- 2.81	-5.65	+ 5.02
Tryptophane	-62.6	+56.6	-59.2	+87.3
Tyrosine	-26.8	-17.0	-26.0	+ 2.68
Valine	-16.6	- 5.86	-15.7	+ 3.22

* Expressed as per cent increase or decrease from the analytical values for the organisms grown on ammonium citrate.

FIGURE 1.

Variation in Amino Acid Content of *Mycobacterium phlei* Cell Proteins with Substrate Nitrogen

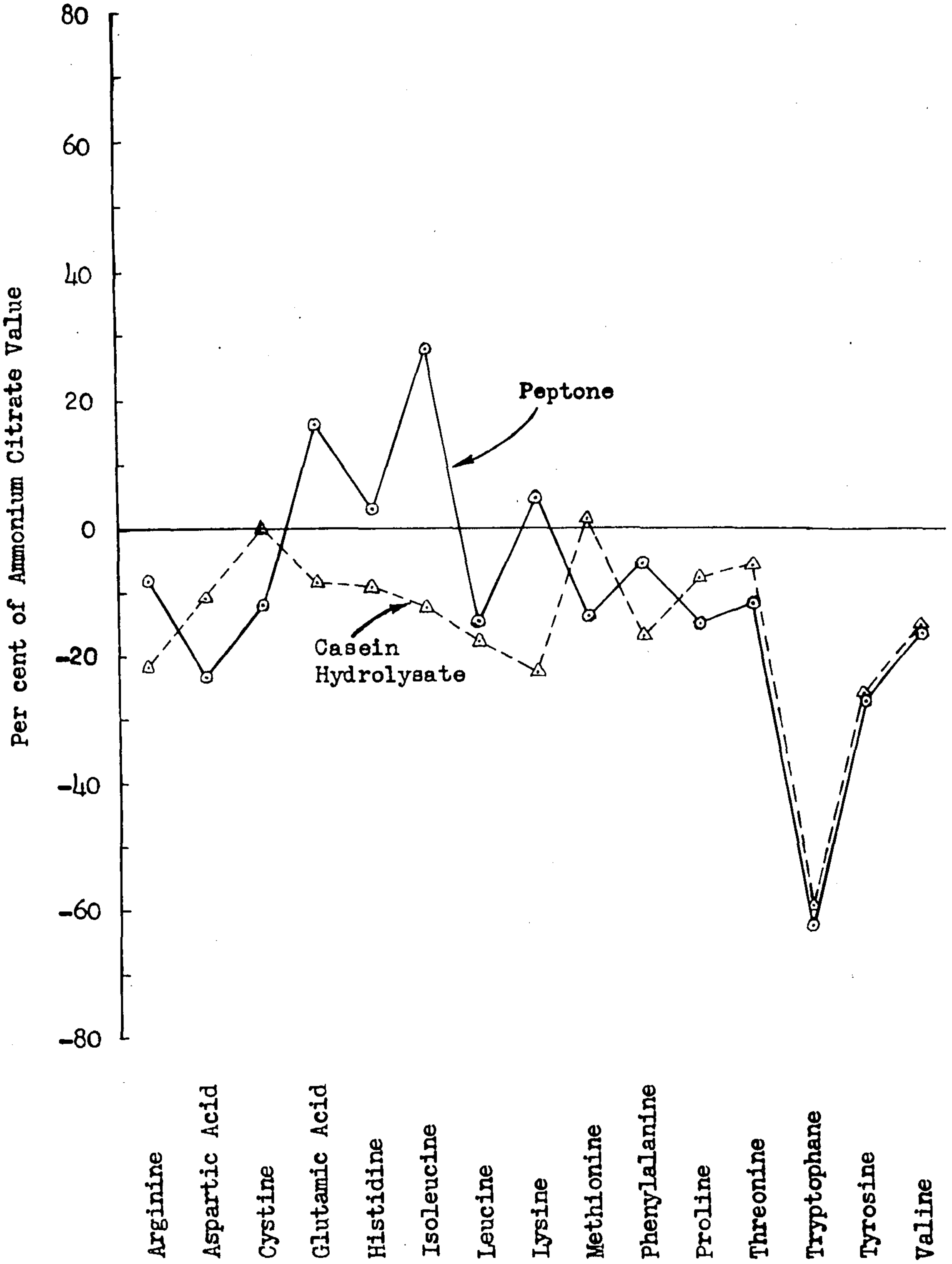
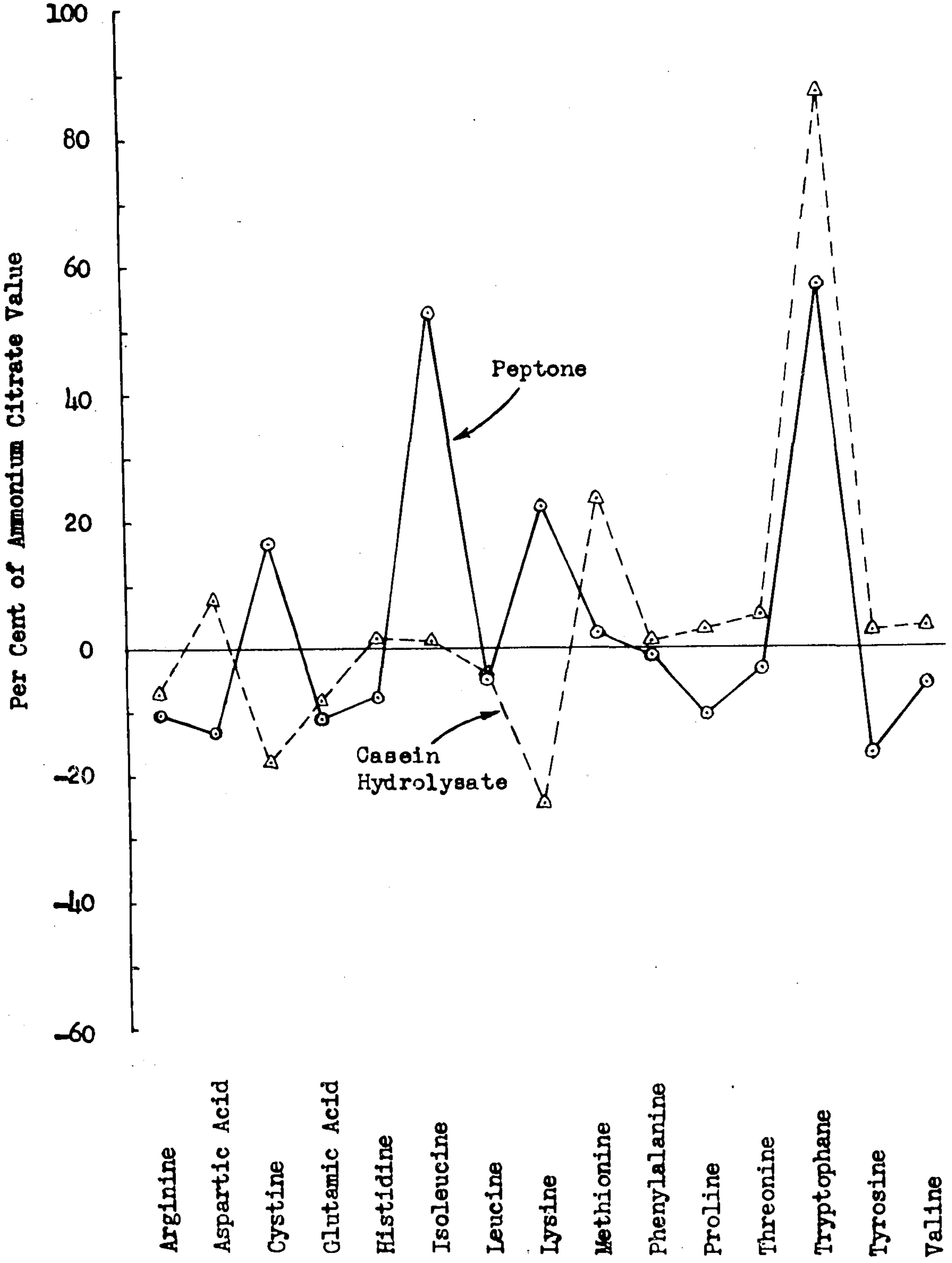


FIGURE 2.

Variation in Amino Acid Content of _Mycobacterium avium_ Cell Proteins with Substrate Nitrogen



DISCUSSION

The results obtained clearly indicate a variation in the amino acid and protein content of each test organism in response to changes in the substrate nitrogen. To illustrate more strikingly the scope of the quantitative changes involved, the variation in content of each amino acid after growth of the organisms upon peptone and casein hydrolysate, respectively, was calculated as per cent increase or decrease from the analytical value found after growth upon ammonium citrate. These per cent variations are recorded in Table VI, and illustrated graphically in Figures 1 and 2. With a few exceptions, each variation is much greater than could be reasonably attributed to the inaccuracy of the particular analytical method. Reference is made to Table III, in which are recorded the ranges of disagreement between duplicate samples, compiled from all the data obtained for each assay procedure.

The protein of a bacterial cell is a complex mixture of a large variety of simple and conjugated proteins. Amino acid analysis of the total cell protein is a procedure which disregards changes in the various proteins unless they are accompanied by quantitative changes in the amino acids. Conceivably, other important changes in the characteristics of the cellular proteins, such as the sizes of the molecules and arrangements or sequences of the amino acids contained therein, could occur while the amino acid and/or protein content of the cell remained ostensibly the same.

However, since significant quantitative differences in the amino acid and protein content were obtained by varying the type of substrate nitrogen, it may be considered that these reflect important modifications in the amount, character and distribution of the cellular proteins. These modifications may be in the structural proteins of the cell as well as the enzymes, although it seems likely that changes in the enzymic constitution were of the greatest importance in the present study. On medium "A" the organisms had to evolve the enzymes necessary for the synthesis of all their constituent amino acids, with an ammonium salt supplying the nitrogen. On medium "B" the organisms presumably evolved proteolytic enzymes to break down the peptone to free amino acids and small peptides, which were then absorbed and utilized in the synthesis of cellular proteins. Casein hydrolysate, a source high in content of free amino acids and small peptides, supplied those substances for immediate absorption when the organisms were grown on medium "C".

Since the organisms would have to produce intracellular enzymes for the synthesis of all their constituent amino acids when grown on the ammonium citrate medium, they presumably would have a higher enzymic, and hence total protein, content than when grown on media complete in all the amino acids. This hypothesis is supported by the observation that the total protein of each organism was significantly less (refer to Tables IV and V) when it was cultured on the peptone medium. In the case of M. phlei the total protein decreased still further when that organism was grown on the casein hydrolysate medium. However, on the same medium the total protein of M. avium was observed to increase significantly to a value only slightly less than that found for the organism grown on ammonium citrate.

From the data presented in Table VI and illustrated graphically in Figures 1 and 2 it is evident that the observed variations in amino acid content do not necessarily parallel one another, even though the organisms are of closely related species. This is true for those changes which occurred when the two organisms were cultured on the same medium, as well as for those changes observed in the same organism grown on two media which differ primarily in the availability of the amino acids supplied.

Of the eleven significant variations (10 % or more for tryptophane, 5 % or more for all others) observed for M. avium and thirteen for M. phlei after growth on the peptone medium, only six were parallel; the arginine, aspartic acid, proline, tyrosine, and valine content decreased, while the isoleucine content increased in both cases. Of the eight significant variations observed for M. avium and thirteen for M. phlei after growth on the casein hydrolysate medium, only three were parallel; the arginine, glutamic acid, and lysine content decreased in both cases.

The concurrent, large increases in content of tryptophane observed for M. avium after growth on the peptone and casein hydrolysate media are of interest, although that amino acid was detected in only small amounts in both organisms throughout the study. The data obtained for M. phlei after growth on the same two media reveal concurrent, significant decreases in content of tyrosine, valine, and tryptophane, which are especially large in case of the latter.

These pronounced differences in the variations of the amino acid content of the two bacilli with the type of substrate nitrogen suggest that fundamental differences may exist between these organisms in the nature of the enzymes and mechanisms involved in their nitrogen metabolism.

SUMMARY

1. Significant quantitative changes in the amino acid and protein content of two related acid-fast bacilli were induced by varying the type of substrate nitrogen.

2. It may be considered that these changes reflect important modifications in the character and distribution of the enzymes involved in the nitrogen metabolism of the two organisms.

3. The pronounced differences observed in the variations of the amino acid content of the two bacilli suggest that fundamental differences may exist between these organisms in the nature of the enzymes and mechanisms involved in their nitrogen metabolism.

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