14013

Amino Acid Requirement of *Lactobacillus casei.*

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During recent years the lactic acid bacterium, *Lactobacillus casei,* has been studied rather extensively with respect to its growth factor requirements. From these studies it has been established that the essential growth factors needed by this organism are: riboflavin,[1] pantothenic acid,[2] nicotinic acid,[2] pyridoxine,[3] biotin,[4] and the eluate factor (folic acid).[3,4,5] The effect of stimulatory factors, that is, factors that the organism can synthesize slowly but which, if added preformed to the medium facilitate growth during early periods, have not as yet been studied in such detail. Glutamine, asparagine, adenine, and guanine have been reported, under certain conditions, as falling into this category.[6]

Recently it was reported that *L. casei* could be subcultured on a medium containing riboflavin, pantothenic acid, nicotinic acid, pyridoxine, biotin, concentrates of the eluate factor, glucose, minerals and acid hydrolyzed casein as a source of amino acids.[4] It is the purpose of this communication to describe the amino acid requirements of *L. casei* on this defined medium.

Method of assay. Assay procedure followed accepted technics. The inoculum was prepared by transferring the organism from a stock culture into a medium of the following composition:

	%
Glucose	2.0
Sodium acetate	0.6
Acid hydrolyzed casein	0.5
Tryptophane	0.01
Cystine	0.01
	mg %
Adenine	2.0
Riboflavin	0.02
Calcium pantothenate	0.02
Nicotinic acid	0.02
Pyridoxine	0.02
Biotin (free acid)	0.00001
Eluate factor concentrate	0.001

Salts per 1000 ml of medium as follows: K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot 4H_2O$, 0.01 g.

After 24 hr incubation at 37°, the cells were centrifuged down and resuspended in 10 ml of sterile 0.9% saline. One ml of this suspension was diluted to 10 ml with sterile saline. One drop of this suspension was

TABLE I.
Effect of Omitting One Amino Acid on Growth.

Amino acid omitted from basal medium*	ml of N/10 acid per 10 ml of medium
None	7.8
Glycine	7.5
dl Alanine	5.8
dl Leucine	0.5
dl Isoleucine	5.2
dl Norleucine	7.4
dl Serine	0.6
dl Threonine	6.7
dl Phenylalanine	0.7
dl Lysine	6.8
dl Methionine	6.6
dl Glutamic acid	0.4
dl Valine	0.5
dl Aspartic acid	0.5
l Cystine	0.4
d Arginine	0.5
l Histidine	5.3
l Tryptophane	0.8
l Proline	7.5
l Hydroxyproline	7.4
l Tyrosine	0.7

*The basal contained all of the constituents listed on this page excepting acid hydrolyzed casein. This was replaced by a mixture of the 20 amino acids given above; 1 mg of each amino acid per 10 ml of medium.

[1] Snell, E. E. Strong, F. M., and Peterson, W. H., *Biochem. J.*, 1937, **31**, 1789.

[2] Snell, E. E., Strong, F. M., and Peterson, W. H., *J. Am. Chem. Soc.*, 1938, **60**, 2825.

[3] Snell, E. E., and Peterson, W. H., *J. Bact.*, 1941, **39**, 273.

[4] Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

[5] Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1941, **63**, 2284.

[6] Feeney, R. E., and Strong, F. M., *J. Am. Chem. Soc.*, 1942, **64**, 881.

added per assay tube. The assay tubes were incubated at 37°. At convenient intervals during the first 30 hr of growth turbidity readings were made with an Evelyn photoelectric colorimeter and at the end of 72 hr incubation the amount of lactic acid formed was determined by titration. As the same relative results were obtained by both methods only the titration values are given. All experiments were run at least 3 times and in some cases 5 times.

In the amino acid experiments, the acid hydrolyzed casein, tryptophane and cystine were replaced by a mixture of 20 amino acids. In the first part of the work (Table I) the level of each amino acid was 1 mg per 10 ml of medium. Later it was found that this concentration was too low to insure good growth and the level was raised to 2 mg per 10 ml of medium (Tables II and III). At this concentration the organism grew just as well as when hydrolyzed casein supplemented with tryptophane and cystine was used as the source of amino acids.

TABLE II.
Effect of Stimulatory Amino Acids.

Amino acid added to basal medium	ml N/10 acid per 10 ml of medium
Basal*	3.2
Threonine	6.3
Threonine + methionine	7.4
20 amino acids	10.8
Hydrolyzed casein	10.9

*Besides glucose, buffer, adenine, vitamins and salts the basal contained leucine, serine, phenylalanine, glutamic acid, valine, aspartic acid, cystine, arginine, tryptophane and tyrosine; 2 mg of each amino acid per 10 ml of medium.

TABLE III.
Antagonistic Relations Among Amino Acids.

Amino acid added to basal medium	ml N/10 acid per 10 ml of medium
Basal*	5.9
Histidine	5.3
Isoleucine	5.3
Lysine	6.6
Alanine	7.6
Histidine + isoleucine	4.9
Histidine + lysine	5.9
Histidine + alanine	7.1
Isoleucine + lysine	5.4
Isoleucine + alanine	7.7
Lysine + alanine	9.6
Histidine + isoleucine + lysine	5.4
Histidine + isoleucine + alanine	7.2
Histidine + lysine + alanine	9.6
Isoleucine + lysine + alanine	11.2
Histidine + isoleucine + lysine + alanine	11.5
Hydrolyzed casein	11.4

*Contained the ten amino acids listed in the footnote of Table II plus threonine and methionine; 2 mg of each amino acid per 10 ml of medium.

The effects of omitting each of the 20 amino acids in turn from the medium but retaining the other 19 are summarized for a typical experiment in Table I. No growth or only slight growth occurred in the absence of leucine, serine, phenylalanine, glutamic acid, valine, aspartic acid, cystine, arginine, tryptophane and tyrosine. Growth was restricted in the absence of alanine, isoleucine, threonine, lysine, methionine and histidine. The need for cystine and tryptophane confirmed the previous report[1] that these 2 amino acids are required by *Lactobacillus casei.*

In the next experiment the effect of adding threonine and methionine to a basal containing the 10 amino acids found to be essential in the previous experiment was determined. The data are given in Table II. With all 10 essential amino acids poor acid production was obtained. Improvement resulted when threonine and methionine were added but the acidity was not equal to that obtainable with 20 amino acids. Experiments were therefore set up to determine how many more amino acids were needed to reach the top level.

When the other stimulatory amino acids were added singly to the above 12 amino acids, certain antagonistic relations were observed. A typical series is given in Table III. Histidine and isoleucine were inhibitory while lysine and alanine were stimulatory. Furthermore, the inhibition of histidine and isoleucine and the stimulation of alanine and lysine were somewhat additive. Lysine counteracted the inhibitory effect of histidine and of isoleucine. Alanine more than reversed the effect of histidine or isoleucine either alone or of the two in combination. Isoleucine, lysine and alanine together produced a marked stimulation and the addition of histidine, making a total of 16 amino acids, increased the growth to that obtained with hydrolyzed casein. The growth on the basal was not equal to that obtained with the same

combination in Table II. Variations occurred from series to series but the relative results within a given series were the same for 5 different trials. Such variations are likely to occur in sub-optimal media and are probably related to the vigor of the organism in a given inoculum and its ability to overcome an unfavorable environment.

Summary. The amino acid requirement of *Lactobacillus casei* was determined on a medium containing glucose, sodium acetate, salts, riboflavin, pantothenic acid, nicotinic acid, biotin, pyridoxine, adenine, and concentrates of the eluate factor (folic acid). The simplest amino acid mixture that would yield maximum growth on repeated subculture was: leucine, serine, phenylalanine, glutamic acid, valine, aspartic acid, cystine, arginine, tryptophane, tyrosine threonine, methionine, alanine, isoleucine, lysine, and histidine.

14014

Effect of Salt and Substrate Concentration upon Activity of Adenosinetriphosphatase.*

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Although the procedure used by some workers for determining adenosinetriphosphatase (ATP-ase) activity[1,2] is adequate for many purposes, such a measurement of the increase in inorganic phosphate at a single time is not necessarily proportional to the enzyme activity. The present studies were undertaken to determine under what conditions the initial rate of formation of inorganic phosphate would be linear, or could be taken as proportional to the enzyme concentration. In the course of the study, it also became evident that the activity of the enzyme was markedly affected by the salt concentration. These observations will also be reported.

Materials. The adenosinetriphosphate (ATP) was prepared from rabbit muscle by the method of Kerr.[3] The dibarium salt of ATP was precipitated twice from water, and was dried over P_2O_5. Although the amount of organic phosphorus was close to the theoretical value, the labile phosphate (readily hydrolyzed with 1 N acid at 100°, and corresponding to 2 moles of phosphate per mole of ATP) indicated that the material was only about 60% pure. The concentrations of ATP given are calculated from the labile phosphate.

For use in the experiments, a weighed amount of the dibarium salt was dissolved in 0.1N HCl and the barium precipitated by the addition of a slight excess of solid Na_2SO_4. The barium sulfate was removed by centrifugation or filtration, the solution neutralized with NaOH, and made up to volume.

The ATP-ase was prepared from rat muscle by the procedure commonly used for the preparation of myosin. Muscle from the hind limb was frozen in dry ice, ground in a Wiley mill, and extracted with 0.5 N KCl. The pH was kept in the neighborhood of 7 to 7.5 during the extraction by the addition of $NaHCO_3$. The extract was centrifuged at a speed of 10,000 RPM in an angle centrifuge. The pH of the supernatant was adjusted to 6.5 with acetic acid, and 7 volumes of cold water added. The myosin precipitate was centrifuged off and washed once with cold water. The myosin was then dissolved with sufficient KCl to make the concentration 0.5 M. The precipitation was repeated twice more, and the myosin was kept in 0.5 M KCl

* This work was aided by a grant from the Rockefeller Foundation.

1 Needham, D. M., *Biochem. J.*, 1942, **36**, 113.

2 Bailey, Kenneth, *Biochem. J.*, 1942, **36**, 121.

3 Kerr, Stanley E., *J. Biol. Chem.*, 1941, **139**, 121.