GROWTH FACTORS FOR BACTERIA

X. Additional Factors Required by Certain Lactic Acid Bacteria[1]

E. E. SNELL and W. H. PETERSON

Departments of Biochemistry and Agricultural Bacteriology, University of Wisconsin, Madison

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Previously reported studies of lactic acid bacteria have shown that these organisms are as complex in their nutritional requirements as any group of microorganisms investigated. Certain of them have been shown to require riboflavin (Snell and Strong, 1939), pantothenic and nicotinic acids (Snell, Strong and Peterson, 1939) and vitamin B_6 (Möller, 1938) in addition to the amino-acid tryptophane and other unidentified constituents of hydrolyzed casein or peptone media (Snell, Tatum and Peterson, 1937), (Snell, Strong and Peterson, 1939).

In attempting to culture a representative organism of the group on a purified hydrolyzed casein medium which contained the known growth requisites, little or no growth was secured unless plant or animal extracts were added to the medium. The nature of the additional factors required for growth, and the concentration of one of them, are the subjects of the present communication.

EXPERIMENTAL

Organism

The organism used is that described as *Lactobacillus casei* in our previous communications. It is the *Bacillus casei* ϵ of Freudenreich (1904), now classified as *Lactobacillus helveticus* (Bergey *et al.*, 1939).

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Medium

The medium was designed to contain all the known requirements of this organism in optimal amounts, together with an adequate supply of amino acids. It had the following composition:

Acid hydrolyzed, purified casein*	0.5 per cent
Sodium acetate (anhydrous)	0.6 per cent
Glucose (monohydrate)	1.0 per cent
Tryptophane	0.01 per cent
Cystine (hydrochloride)	0.01 per cent
Riboflavin	0.01 mgm. per cent
Nicotinic acid	0.02 mgm. per cent
Pantothenic acid†	0.05 mgm. per cent
Mineral salts‡	

* 100 grams of Labco Casein, Vitamin-Free (Casein Company of America, New York, N. Y.) was hydrolyzed by heating in the autoclave at 15 pounds pressure with 375 ml. of water and 125 ml. of concentrated H_2SO_4. The hydrolysate was diluted, and SO_4^- removed with $Ba(OH)_2$.

† The preparation made as previously described (Snell, Strong and Peterson, 1939) contained about 5 per cent pantothenic acid.

‡ Mineral salts were used in the following concentrations. K_2HPO_4, 0.5 grams; KH_2PO_4, 0.5 grams; $MgSO_4 \cdot 7H_2O$, 0.2 grams; NaCl, 0.01 grams; $FeSO_4 \cdot 7H_2O$, 0.01 grams; $MnSO_4 \cdot 3H_2O$, 0.01 grams; in 1000 ml. of medium.

The medium was made up in twice the above concentration; adjusted to pH 6.8, and tubed in 5 ml. lots. Fractions to be tested for supplementary activity were then added, the final volume was made up to 10 ml.; the tubes were plugged with cotton and sterilized in the autoclave at 15 pounds pressure for 15 minutes.

Inoculum

The inoculum used was a 24-hour culture of *Lactobacillus casei* grown in Medium B (Snell, Strong and Peterson, 1937) supplemented with 5 micrograms of the pantothenic acid preparation described above. The organisms were centrifuged from 10 ml. of culture medium, and re-suspended in 100 ml. of sterile 0.9 per cent saline solution. One drop of the resulting suspension (barely visible turbidity) was used to inoculate each tube. Use of a larger inoculum led to considerable growth in the unsupplemented controls. This, however, did not continue on subculture.

Incubation

Cultures were incubated at 37°C. for 48 hours. Growth was measured by titration of acid produced. Acid production continues slowly for 96 hours, but for purposes of assay the two-day period was very satisfactory.

Effect of yeast on growth in the basal medium

Of several substances tested, yeast extract (Bacto) proved most potent in promoting growth on the above medium. Its fractionation was therefore begun. It was soon observed, however, that no single substance was responsible for the stimulating effect of the extract. At least two distinct substances were required. These were separated by selective adsorption as follows:

TABLE 1

Supplementation of basal medium by yeast extract; separation into two essential fractions

SUPPLEMENT TO BASAL MEDIUM	MGM. OF SUPPLEMENT ADDED PER 10 ML.			ML. N/10 ACID PRODUCED PER 10 ML.		
	(a)	(b)	(c)	(a)	(b)	(c)
None				0.6	0.7	
Yeast extract	0.5	1	3	5.5	8.0	10
Norite filtrate	1	1	3	0.8	0.8	1.5
Norite eluate	0.01	0.03	0.05	1.8	2.2	2.5
Norite filtrate + Norite eluate	1 0.01	1 0.03	1 0.05	3.0	5.8	6.9

Five grams of Bacto yeast extract were dissolved in 100 ml. of water. Sulfuric acid was added to pH 3.0, then 2 grams of Pfanstiehl Norit A was added and the mixture stirred for one-half hour. The norite was centrifuged out and eluted once with 50 per cent ethanol. This eluate was discarded. The norite was then eluted 3 times with a pyridine-ethanol-water mixture (1:2:1) and the eluate concentrated in vacuum to dryness. The norite filtrate and eluate fractions were assayed separately and together for their growth-promoting activity. Results are summarized in table 1. The supplementary action of the norite filtrate when combined with the eluate is clearly observable.

Thus, it appears that this organism requires two separate factors in yeast, not supplied by the basal medium. One of these is very easily adsorbed on norite; the other is less readily adsorbed. These will be referred to subsequently as the norite eluate and the norite filtrate factors, respectively.

Effect of vitamin B_6[2]

Since Möller (1938) has reported vitamin B_6 to be essential for certain lactic acid bacteria, its effect in replacing the norite eluate and the norite filtrate was tested. Results are given in table 2.

TABLE 2

Effect of vitamin B_6 on the growth of Lactobacillus casei

SUPPLEMENT TO BASAL MEDIUM	MGM. SUPPLEMENT ADDED PER 10 ML. MEDIUM				ML. N/10 ACID PRODUCED PER 10 ML. MEDIUM			
	(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)
None					1.0	0.9		
Norite filtrate	1	1	2	2	1.3	1.3	1.8	1.7
Vitamin B_6	0.001	0.003			1.1	1.1		
Norite eluate	0.01	0.01	0.02	0.02	2.1	2.1	2.4	2.4
Norite filtrate + Vitamin B_6	1 0.001	1 0.001	2 0.001	2 0.001	1.3	1.4	1.8	1.8
Norite eluate + Vitamin B_6	0.01 0.001	0.02 0.0001	0.02 0.001	0.02 0.003	3.3	3.5	4.5	4.3
Norite eluate + Norite filtrate	0.01 1	0.01 2	0.02 1	0.02 2	3.4	4.1	5.0	7.8

Vitamin B_6 was ineffective in replacing the norite eluate, but was highly effective in replacing the norite filtrate factor. We are thus able to confirm the report of Möller that vitamin B_6 is required by some lactic acid bacteria. Examination of the last two lines of the table, however, will show that the effect of vitamin B_6 reached a maximum at a titration of 4.4 ml., while the norite filtrate permitted considerably greater growth. It is highly probable therefore, that the norite filtrate contains a factor (or factors) other than vitamin B_6 which stimulates the growth of this organism.

[2] We wish to thank Dr. S. Lepkovsky for gifts of crystalline vitamin B_6.

Assay of the norite eluate factor

It was decided to investigate the nature of the norite eluate more closely. Separate experiments showed that it could not be replaced by a mixture of known compounds with growth factor activity (pimelic acid, uracil, β-alanine, inositol, betaine, thiamin, and a preparation of the acid growth factor for butyl alcohol anaerobes (McDaniel *et al.*, 1939)).

TABLE 3

Potencies of various source materials in the norite eluate factor

SUPPLEMENT TO BASAL MEDIUM	MGM. SUPPLEMENT ADDED PER 10 ML. MEDIUM				ML. N/10 ACID PRODUCED PER 10 ML. MEDIUM				WEIGHT OF ONE UNIT
	(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)	
									mgm.
None					0.7	0.8			
Bacto yeast extract	0.1	0.3	0.5	1.0	2.3	5.0	6.2	7.6	0.22
Alcohol soluble, liver fraction*	0.5	1.0	3.0	5.0	1.3	2.0	4.2	6.3	2.7
Solubilized liver fraction*	0.05	0.1	0.3	0.5	3.8	6.8	7.8	7.9	0.05
Vitab†	0.5	1.0	3.0	5.0	1.3	1.8	3.3	4.8	3.7
Liver extract (aqueous)	0.1	0.3	0.5	1.0	1.6	3.0	4.2	6.5	0.43
Malt sprouts extract	0.1	0.3	0.5	1.0	2.2	4.8	6.2	7.5	0.22

* We wish to thank Dr. David Klein, of the Wilson Laboratories, Chicago, for these preparations. The alcohol-soluble fraction represents that portion of an aqueous liver extract soluble in 92 per cent ethanol. The solubilized liver fraction is that portion of an aqueous liver extract precipitated from solution by addition of ethanol to 70 per cent concentration, then rendered water-soluble by enzyme action.

† A commercial extract of cereal grains. We are indebted to the Vitab Corp., Emeryville, Calif., for this material.

For the routine assay of this substance the following substances were added to the basal medium above:

Norite filtrate of yeast extract (prepared as above and dried) ..... 0.025 per cent
Vitamin B_6 ..... 0.02 mgm. per cent

Although the norite filtrate contained some of the necessary B_6, this substance became a factor limiting growth at higher concentrations of the norite eluate, and was, therefore, added to the medium in pure form. With these additions to the medium, the assay was carried out as described above.

Sources of the norite eluate factor

The comparative potencies in the norite eluate factor of a variety of preparations is shown in table 3. The solubilized liver fraction was by far the best source of this material. The table illustrates well the type of data secured by the assay. For quantitative assay, one unit is taken as that amount of material giving approximately half-maximum fermentation. This is about equal to the effect of 0.05 mgm. (50 micrograms) of the solubilized liver fraction. To eliminate variation in the fermentation, the unit has been taken as that acidity produced by 50 micrograms of the solubilized liver fraction.

Fractionation of the norite eluate factor

Because of its potency and availability, the solubilized liver fraction has been used as a starting material. The fractionation procedure so far worked out is illustrated by the following run:

a. *Adsorption and elution from norite.* Five-hundred grams of the solubilized liver fraction was dissolved in 10 liters of water. Sulfuric acid was added to pH 3.0, and the flocculent precipitate filtered off. To the filtrate was added 100 grams of Pfanstiehl Norit A; the mixture was stirred for one-half hour, then filtered (filtrate = Fraction A), and the norite washed with water. The norite was then suspended in 1 liter of 50 per cent ethanol, stirred for 1 hour and filtered (filtrate = Fraction B). The norite was then eluted 3 times by stirring for one-half hour each time with 600 ml. of pyridine-ethanol-water (1:2:1). The combined pyridine eluates were concentrated to dryness in vacuum (Fraction C).

	WEIGHT	1 UNIT	TOTAL UNITS	PERCENTAGE OF ACTIVITY RECOVERED
Solubilized liver fraction....	500 grams	50 micrograms	10,000,000	
Fraction A.....	10 l.	0.02 ml.	500,000	5.0
Fraction B.....	12 grams	125 micrograms	96,000	0.96
Fraction C.....	27 grams	5.8 micrograms	4,650,000	46.5

About 50 per cent of the activity is recovered in all; evidently the elution from norite is far from complete. Recent work has shown that quantitative elution from norite can be effected by the use of 0.3 to 2.0 per cent aqueous ammonia. Thus a run made just as above, but the final elution carried out in two steps with 2 per cent aqueous ammonia, yielded (from 500 grams of starting material) 13.2 grams of eluate, containing one unit in 1.25 micrograms, or an activity recovery of 105 per cent. Further fractionation of this more promising material by the procedures given below has not been carried out.

b. *Extraction with butyl alcohol.* The active material was not extracted by ether from aqueous solution at acid, neutral or alkaline reaction. Butyl alcohol extracted the active material at pH 4.0, but failed to extract it at pH 7.5 to 8.0 or at pH 10.0. Some purification resulted from the extraction, so this material was incorporated in the procedure. A typical experiment follows.

Four grams of Fraction C (above) was dissolved in 80 ml. of water. The pH of the solution was 4.0. It was extracted continuously with butyl alcohol under reduced pressure for 48 hours, and the extract (Fraction D) and unextracted material (Fraction E) were dried and assayed.

	WEIGHT	1 UNIT	TOTAL UNITS	PERCENTAGE OF ACTIVITY RECOVERED
	grams	*micrograms*		
Fraction C	4	5.8	690,000	
Fraction D	1.3	2.3	565,000	82
Fraction E	2.6	22.5	115,000	16.7

c. *Precipitation with picric acid.* One gram of a butyl alcohol extract corresponding to Fraction D, but somewhat less active (1 unit; 3.2 micrograms) was dissolved in 10 ml. of water, and warmed to 80°. Ten milliliters of a saturated (50°) solution of picric acid were added, and the mixture placed in the refrigerator over night. The insoluble picrates were separated from the water soluble picrates. Excess picric acid was removed from the latter with ether, and the remaining picrates were concentrated to dryness (Fraction F).

The water-insoluble picrates were separated into alcohol-soluble and alcohol-insoluble components by boiling with 10 ml. of 95 per cent ethanol. The alcohol-insoluble portion was dried, weighed, dissolved in a large volume of boiling water and diluted for assay (Fraction G). The deep red alcohol solution was concentrated to dryness, and the residue leached out twice with ether. The ether-soluble fraction (Fraction H) and the ether-insoluble fraction (Fraction I) were dissolved in alcohol and diluted with water for assay.

	WEIGHT	1 UNIT	TOTAL UNITS	PERCENTAGE OF ACTIVITY RECOVERED
	grams	*micrograms*		
Butyl alcohol extract (similar to fraction D)	1.00	3.2	312,000	
Fraction F	0.75	15.5	48,400	15.5
Fraction G	0.092	12.0	7,700	2.5
Fraction H	0.051	Inactive		
Fraction I	0.078	0.55	142,000	45.5

The water and ether-insoluble, alcohol-soluble picrates contained the bulk of the activity. Considerable loss attends the procedure. Picric acid is non-toxic in concentration equal to that added with these fractions for assay. Fraction I is the most active preparation so far obtained.

Miscellaneous properties of the norite eluate factor

a. *Stability to acid and alkali.* In the crude condition (yeast extract, 1 unit = 0.22 mgm.), the factor was unharmed by autoclaving for 1 hour at pH values from 1 to 13. An extract (1 unit = 50 micrograms) was $\frac{5}{6}$ destroyed (1 unit = 300 micrograms) by autoclaving for $1\frac{1}{2}$ hours with 3N NaOH, however, and was completely destroyed by autoclaving $1\frac{1}{2}$ hours with 5N HCl.

b. *Treatment with acetic anhydride.* One-hundred fifty milligrams of a preparation (1 unit = 3.8 micrograms) was heated on the steamer for 1 hour with 1 ml. of acetic anhydride and 1 ml. of pyridine. The product, which was not completely soluble in water, was inactive. A control, heated with glacial acetic acid

and pyridine showed no decrease in activity. Attempts to reactivate the acetylation product by hydrolysis with saturated methyl-alcoholic ammonia in a sealed tube at 100° for 1 hour, or by heating for one-half hour with 1N NaOH failed completely.

c. *Stability to hydrogen peroxide.* Fifteen milligrams of the factor (1 unit = 32.5 micrograms) was heated in the steamer for one hour with 1 ml. of 3 per cent hydrogen peroxide. The decolorized solution was completely inactive.

d. *Precipitation by phosphotungstic acid.* One gram of a norite eluate (1 unit = 1.65 micrograms) was dissolved in 10 ml. of 5 per cent sulfuric acid and a 30 per cent solution of phosphotungstic acid in 5 per cent sulfuric acid added until precipitation was complete. The precipitate was centrifuged out. The supernatant liquid, containing the unprecipitated material, was extracted continuously with ether for 2 days to remove the phosphotungstic acid; the sulfuric acid was then exactly removed with barium hydroxide, and the filtrate concentrated to dryness (Fraction J).

The precipitate was desiccated, pulverized, and shaken with 30 ml. of acetone for 1 hour, filtered, and the extraction with acetone repeated. The acetone-soluble phosphotungstates (Fraction K) and the acetone-insoluble phosphotungstates (Fraction L) were assayed directly without decomposition by dissolving in a large volume of hot water and diluting until no precipitate formed on cooling.

	WEIGHT	1 UNIT	TOTAL UNITS	PERCENTAGE OF ACTIVITY RECOVERED
	grams	*micrograms*		
Norite eluate	1	1.65	606,000	
Fraction J	0.252	Inactive		
Fraction K	0.708	Inactive		
Fraction L	2.46	8.7	283,000	47

The active substance is evidently precipitated by phosphotungstic acid, and its phosphotungstate is insoluble in acetone. Most amino-acid phosphotungstates and amine phosphotungstates are very soluble in acetone; insolubility in this solvent is

characteristic of the purine phosphotungstates (Drummond, 1918; Wechsler, 1911). In this case, as with the picrates, it is possible that the apparent low yield in activity is due to lessened availability of the salt combination to the microorganisms.

e. *Precipitation by basic lead acetate.* To 25 mgm. of a norite eluate (1 unit = 5.2 micrograms) in 5 ml. of water (pH 3.4) was added a solution of 40 mgm. of basic lead acetate. The precipitate formed was centrifuged out. Lead was removed from filtrate and precipitate by adding an excess of M/5 K_2HPO_4 and adjusting the pH to 7.6. The precipitated lead phosphate was centrifuged out, and the two fractions each diluted to 25 ml. and assayed.

	1 UNIT	TOTAL UNITS
Starting material	5.2 micrograms	4,800
Lead filtrate	inactive	
Lead precipitate	0.0052 ml.	4,800

The active material was quantitatively precipitated by basic lead acetate.

f. *Precipitation by copper sulfate and sodium bisulfite.* 0.5 gram of a norite eluate (1 unit = 1.65 micrograms) was dissolved in 5 ml. of water. To the solution was added 10 ml. of a solution containing 0.5 gram $CuSO_4 \cdot 5H_2O$ and 0.5 gram $NaHSO_3$. A very heavy green precipitate was obtained. This was digested in a boiling water bath until it became granular and assumed a brownish red color. This was centrifuged out. Cu^{++} was removed from filtrate and precipitate with H_2S.

	WEIGHT	1 UNIT	TOTAL UNITS	PERCENTAGE OF ACTIVITY RECOVERED
	grams			
Norite eluate	0.5	1.65	303,000	
Copper filtrate		Inactive		
Copper precipitate	0.112	1.37	82,000	27

The material was precipitated by this procedure. The low recovery of activity is attributed to adsorption of the active compound by copper sulfide (cf. below). This procedure has

been recommended (D. Krüger, 1896; cf. Graff and Maculla, 1935) as a specific method for the precipitation of purine bases.

g. *Adsorption on various materials.* In the course of experiments on the lead precipitation described above, it was observed that the active substance was adsorbed very tenaciously from acid solution by lead sulfide. It is adsorbed to a less extent on copper, silver, and zinc sulfides. It is adsorbed from acid solution by excess fuller's earth (Lloyd's reagent was used). As is evident from the fractionation procedure, it is strongly adsorbed by norite at acid pH. At pH above 7.0, however, it is not appreciably adsorbed by norite (cf. the elution from norite by aqueous ammonia).

DISCUSSION

From the evidence presented above, certain deductions concerning the nature of the norite eluate factor may be drawn. Since it is not extracted by butyl alcohol from alkaline solution, but is extracted at weakly acid reaction, the material has some acidic properties. These are, however, far outweighed by its basic nature, which permits its precipitation by picric and phosphotungstic acids. The insolubility of its phosphotungstate in acetone, and its precipitability by copper sulfate and bisulfite under conditions fairly specific for the precipitation of purines, suggest that it may be a member of this or a related group of compounds.

Since the compound appeared to be rather strongly basic, and possibly of a purine nature, a number of compounds were tested for their growth-promoting power on the test organism in addition to those mentioned in the first part of this paper. These included arginine, glutathione, choline, cholamine, hemin, adenylic acid, adenine, guanine, xanthine, hypoxanthine, uric acid, uracil and cytosine. None of these compounds showed any growth-promoting properties under the conditions of the test.

The purest preparation obtained produced half-maximum fermentation of the organism in a concentration of 0.055 microgram per milliliter. Riboflavin and pantothenic acids, two

known accessory factors for this organism, produce half-maximum effect in concentrations of 0.015 microgram per milliliter and 0.006 microgram per milliliter (calculated), respectively. If the new factor has approximately the same activity, the purest concentrates so far obtained would be from 10 to 25 per cent pure.

SUMMARY

The finding of Möller (1938) that vitamin B_6 is required by certain lactic acid bacteria is confirmed. In addition, evidence is presented for the existence of two additional unidentified growth factors required by certain of these organisms for growth in a medium containing amino acids and the known growth factors. One of these has been considerably purified, and certain of its properties described. It is shown to be of basic nature, labile to oxidation, precipitated by many of the common basic precipitants; and it shows some properties in common with the naturally occurring purines.

REFERENCES

Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P. 1939 Bergey's Manual of determinative bacteriology. Fifth Edition, p. 365. The Williams & Wilkins Co., Baltimore.

Drummond, J. C. 1918 Observations on the phosphotungstates of certain bases and amino-acids. Biochem. J., **12**, 5–24.

Freudenreich, E., and Thöni, T. 1904 Über die Wirkung verschiedener Milchsäurefermente auf die Käsereifung. Landw. Jahrb. Schweiz., **18**, 525–548.

Graff, S., and Macullа, A. 1935 The composition of tissue proteins, I. The estimation of purines in tissues. J. Biol. Chem., **110**, 70–80.

Krüger, M. 1896 Die gewinnung des Adenins aus Theextract. Z. physiol. Chem., **21**, 274–284.

McDaniel, L. E., Woolley, D. W., and Peterson, W. H. 1939 Growth factors for bacteria. VII. Nutrient requirements of certain butyl-alcohol-producing bacteria. J. Bact., **37**, 259–268.

Möller, E. F. 1938 Vitamin B_6 (Adermin) als Wuchsstoff für Milchsäurebakterien. Z. physiol. Chem., **254**, 285–286.

Snell, E. E., and Strong, F. M. 1939 The effect of riboflavin and of certain synthetic flavins on the growth of lactic acid bacteria. Enzymologia, **6**, 186–193.

Snell, E. E., Strong, F. M., and Peterson, W. H. 1937 Growth factors for bacteria. VI. Fractionation and properties of an accessory factor for lactic acid bacteria. Biochem. J., **31**, 1789–1799.

SNELL, E. E., STRONG, F. M., AND PETERSON, W. H. 1939 Growth factors for bacteria. VIII. Pantothenic and nicotinic acids as growth factors for lactic and propionic acid bacteria. J. Bact., **38**, 293–308.

SNELL, E. E., TATUM, E. L., AND PETERSON, W. H. 1937 Growth factors for bacteria. III. Some nutritive requirements of *Lactobacillus delbrückii*. J. Bact., **33**, 207–225.

WECHSLER, E. 1911 Zur Technik der Phosphorwolframsäurefällungen. Z. physiol. Chem., **73**, 138–143.