SOME ADDITIONAL NUTRITIONAL REQUIREMENTS OF CERTAIN LACTIC ACID BACTERIA[1]

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As a result of extensive previous work, the nutritional requirements of many lactic acid bacteria are known in considerable detail. Most of the presently known B vitamins and amino acids are required by one or another of the organisms of this group, and such organisms have been extensively applied to the quantitative determination of these substances (reviews: Snell, 1948, 1950).

Despite this large area of information, many lactic acid bacteria still cannot be grown in the absence of crude materials that supply nutritive substances of undefined nature (Rogosa, Tittsler, and Geib, 1947; Kitay, McNutt, and Snell, 1950). In addition to amino acids and the better known B vitamins, isolated representatives of this group of organisms have been shown recently to require unsaturated fatty acids (Hutchings and Boggiano, 1947; Williams, Broquist, and Snell, 1947), pyridoxamine phosphate (McNutt and Snell, 1948, 1950), lactose (Snell, Kitay, and Hoff-Jorgensen, 1948; Shapiro, Rhodes, and Sarles, 1949), vitamin B_{12} (e.g., Shorb, 1948; Skeggs *et al.*, 1948), and various desoxyribosides (Kitay, McNutt, and Snell, 1949, 1950; McNutt and Snell, 1950; Hoff-Jorgensen, 1949). In addition to these pure compounds, unidentified growth factors such as the *Lactobacillus bulgaricus* factor (Williams, Hoff-Jorgensen, and Snell, 1949) and the *Leuconostoc citrovorum* factor (Sauberlich and Baumann, 1948) are essential for certain strains of lactic acid bacteria.

In view of these results, a survey was undertaken to determine the requirement of about thirty strains of lactic acid bacteria for these newly discovered growth factors. All of these organisms, which belonged to seven different species, failed to grow in media lacking certain supplements discussed above. Results showing the requirement of several of these cultures for vitamin B_{12} or for various desoxyribosides, and conditions under which this requirement is eliminated by reducing agents, were presented and discussed by Kitay, McNutt, and Snell (1950). Additional information relative to other nutritional requirements is discussed below.

EXPERIMENTAL RESULTS

Stock cultures, basal media, and experimental procedures were those employed in the preceding paper (Kitay, McNutt, and Snell, 1950). The concentrations of inorganic salts were those found most favorable for growth of each strain

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and are given with the cultures used in table 1. All of the organisms examined grew in this basal medium when it was supplemented with thymidine and filtered tomato juice. The requirement of many of these cultures for desoxyribosides or vitamin B_{12} was determined in the presence of tomato juice (Kitay, McNutt, and Snell, 1950). In preliminary experiments, a mixture of pyridoxamine phosphate and a concentrate of the *Lactobacillus bulgaricus* factor (LBF; Williams, Hoff-Jorgensen, and Snell, 1949) duplicated the growth-promoting action of the tomato juice for all organisms tested. To determine which of these supplements was essential for growth, a loop transfer from the milk stock culture was made to tubes of the basal medium supplemented only with thymidine (50 μg per 100 ml). Sufficient carry-over of growth factors occurred to permit growth of all cultures in this inoculum tube. Subcultures were then made into 10 ml of this thymidine-supplemented medium with: A, no additional supplements; B, 30 μg of an LBF concentrate;[2] C, 0.02 μg of pyridoxamine phosphate; D, LBF + pyridoxamine phosphate; and E, 1 ml of filtered tomato juice. If growth was obtained in A, this tube was used as inoculum for a second subculture into all five media, and similar subcultures were repeated when necessary until it became evident whether or not additional factors were needed. From the comparative growth in these various media it became apparent whether these supplements were essential or nonessential for growth. The results are presented in table 1.

Similar experiments were conducted to determine whether oleic acid and an enzymatic digest of casein were essential for growth. For this purpose either oleic acid or the enzymatic digest of casein was omitted from the basal medium, which was supplemented with optimum amounts of thymidine, LBF, or pyridoxamine phosphate, as required. In experiments with the enzymatic casein digest, the acid-hydrolyzed casein of the medium was replaced by a complete mixture of amino acids (Henderson and Snell, 1948). Graded amounts of these supplements were then added and the effects on growth noted. The results are summarized in table 1 and discussed briefly below.

Oleic acid. Previous investigations showed this fatty acid to be required by isolated strains of most of the species found to require it here. The data of table 1 show the requirement for it to be widespread. All cultures of *Lacotobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, and *Lactobacillus leichmannii* examined required it. The basal medium upon which this requirement was determined contains an excess of biotin; the presence of biotin does not, therefore, permit synthesis of this fatty acid by these organisms, as it does in many other species of bacteria (Williams, Broquist, and Snell, 1947). Wherever investigated, the requirement for oleic acid has not been specific, since other unsaturated fatty acids of appropriate structure (e.g., linoleic, linolenic, or palmitoleic acids) also permit growth.

Throughout this investigation, "tween 40" was used as a detoxicant for oleic

[2] Concentrates of LBF were prepared by a modification of published procedures (Williams, Hoff-Jorgensen, and Snell, 1949) and were from 143 to 1,200 times as effective on a weight basis in promoting growth as a standard yeast extract.

acid (Williams *et al.*, 1947). For certain cultures that did not require oleic acid for growth in the absence of "tween 40," e.g., *Lactobacillus lactis*, the latter substance proved toxic and its toxicity was eliminated by the addition of oleic acid.

TABLE 1

The requirements of several lactic acid bacteria for oleic acid, thymidine, pyridoxamine phosphate, LBF, and an enzymatic digest of casein

ORGANISMS*	NO. OF CULTURES REQUIRING/NO. OF CULTURES TESTED				
	Oleic acid	Thymidine, other desoxyribosides, or vitamin B_{12}†	Pyridoxamine phosphate	LBF	Enzymatic casein digest‡
Lactobacillus acidophilus	10/10	9/10	1/9	6/9	0/10
Lactobacillus bulgaricus	6/6	0/6	0/6	6/6§	4/6
Lactobacillus delbrueckii	3/3	3/3	1/3	2/3	0/3
Lactobacillus helveticus	3/3	1/3	1/3	1/2	1/3
Lactobacillus lactis	0/2	1/2	0/2‖	0/2	2/2
Lactobacillus leichmannii	3/3	3/3	0/3	0/3	0/3
Leuconostoc citrovorum	0/1	1/1	0/1	0/1	0/1

* Cultures were obtained from Dr. R. P. Tittsler, the American Type Culture Collection, and Dr. W. B. Sarles. In the accompanying list the medium number refers to the salt concentration used in the basal medium for the various organisms, as listed in table 2 of the preceding paper (Kitay, McNutt, and Snell, 1950); the unbracketed numbers following the species names refer to the strain numbers. The numbers in parentheses are those of the American Type Culture Collection. Medium 1: *L. acidophilus* S, *L. helveticus* S; Medium 2: *L. acidophilus* 200, 203 (314), 204 (332), 206 (4357), 213 (4355), (4356), and (832), *L. bulgaricus* 5 (7993) and 14, *L. delbrueckii* 730 (9649), (4796), and (4913), *L. helveticus* 77; Medium 3: *L. acidophilus* 207 (4962) and 217 (4857), *L. bulgaricus* 2 (521), 10 (8018), (7994), and (8001), *L. helveticus* 80, *L. leichmannii* 313 (7830), 326 (4797), and 327 (7831); Medium 4: *Leuconostoc citrovorum* (8081), *L. lactis* 104 and 108.

† The specificity of this requirement is treated in detail in the earlier paper (Kitay, McNutt, and Snell, 1950).

‡ The rate and extent of growth of all organisms tested except *Leuconostoc citrovorum* were stimulated by small amounts of this digest; it was *essential* for growth only as indicated.

§ Several of these strains initiated growth in the control medium without LBF after 72 to 144 hours. When an LBF concentrate was added to this medium, heavy growth was observed in 48 to 72 hours. In subsequent subcultures, growth occurred in shorter incubation periods, i.e., 48 to 72 hours in the control medium and 25 to 48 hours when LBF was present.

‖ Pyridoxamine phosphate, though not essential, markedly stimulated growth of these organisms.

Thymidine, other desoxyribosides, and vitamin B_{12}. The specificity of this requirement has been fully discussed (Kitay, McNutt, and Snell, 1950). These factors are widely required. The lack of requirement for any of this group of substances by *L. bulgaricus* is in marked contrast to the behavior of the other cultures examined.

Pyridoxamine phosphate. The identification of this conjugated form of vitamin B_6 as an essential growth factor for certain of these organisms was recently reported by McNutt and Snell (1948, 1950). The requirement, which is not a common one, is evident in media that contain an excess of the free vitamin. Under appropriate conditions, addition of D-alanine eliminates this requirement for organisms so far tested (McNutt and Snell, 1950; Holden and Snell, 1949).

LBF (*Lactobacillus bulgaricus factor*). A concentrate of this factor was essential for the growth of all strains of *L. bulgaricus* tested, and for most strains of *L. acidophilus* and *L. delbrueckii* (table 1). Since the growth-promoting substance present in these concentrates has not been obtained in pure form, the possibility that more than a single factor was supplied must be considered. To obtain evidence on this point, concentrates of widely varying activity for *L. bulgaricus*

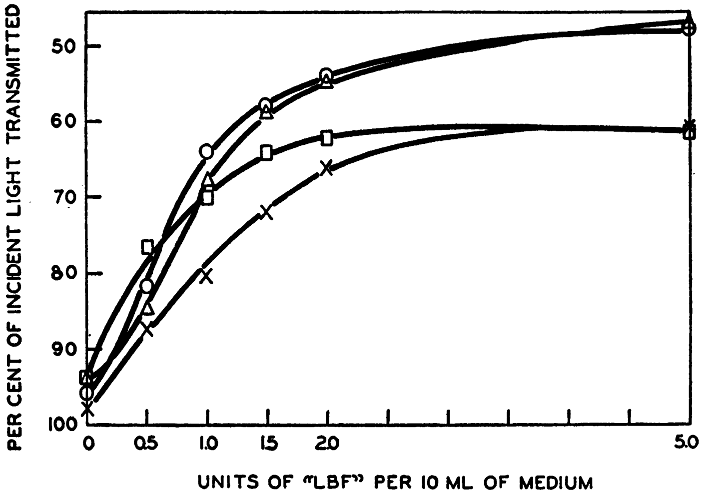

Figure 1. Comparative response of several lactic acid bacteria to a concentrate of LBF. ×——×, *L. acidophilus* 200; □——□, *L. acidophilus* 213; ○——○, *L. acidophilus* 832; △——△, *L. delbrueckii* 4796. The LBF concentrate was 143 times more potent than crude yeast extract and contained 1 unit in 7.0 μg. Growth was estimated after 18 hours of incubation.

Gere A (the standard test strain employed by Williams *et al.*, 1949) were compared in activity for each of the cultures that required this factor. The relative activities of these concentrates were 1.0 (crude yeast extract), 143, and 1,200; the weights of each required to supply 1 unit of activity were 1 mg, 7.0 μg, and 0.83 μg, respectively. Similar dose-response curves, in terms of units of activity, were obtained for all the organisms, a result that indicates strongly that the same substance was promoting growth in each instance. Typical dose-response curves for four different organisms are shown in figure 1.

It is evident from these results that this unidentified biocatalyst, LBF, is required by a great many fastidious bacteria of the lactic group. Organisms that do not require this substance preformed are known to synthesize it (Rasmussen *et al.*, 1950); consequently, the factor must be of general metabolic importance for all organisms.[3]

Enzymatic digest of casein. Enzymatic digests of casein or of other proteins are known to supply one or more factors stimulatory to growth of *Lactobacillus casei* and some other organisms ("strepogenin," Sprince and Woolley, 1945; Wright and Skeggs, 1944). To supply a possible requirement for factors of this nature, such a digest was included in the basal medium throughout our early studies of the nutritive requirements of these cultures. Following identification of oleic acid, desoxyribosides or vitamin B_{12}, pyridoxamine phosphate, and LBF as growth essentials, it became possible to add these substances as pure compounds or as concentrates and thus to determine the role played by the

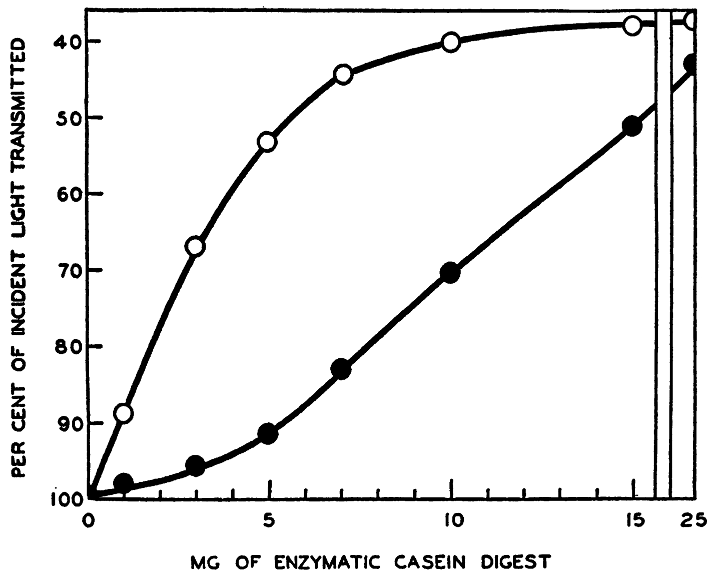

Figure 2. The response of *L. lactis* 104 to additions of an enzymatic digest of casein. ●——●, incubation time, 42 hours; ○——○, incubation time, 89 hours.

enzymatic digest of casein. When this was done, the latter was found to be *essential* for the growth of many cultures of *L. bulgaricus* (e.g., strains 2, 5, 14, and (7994)) and of *L. lactis* (104, 108), and for *L. helveticus* 77. In amounts up to 25 mg per 10 ml it stimulated the growth rate of all of the other cultures tested except *Leuconostoc citrovorum.* At higher concentrations it became somewhat toxic to some of these organisms.

[3] Recent unpublished data (G. M. Brown, J. A. Craig, and E. E. Snell, Arch. Biochem., in press) indicate that LBF is a bound form of pantothenic acid. Many of the organisms listed here as requiring LBF can be grown without it if very high amounts (30 to 50 μg per 10 ml) of calcium pantothenate are added to the basal medium. The amounts of pantothenic acid required to permit growth of such organisms are far higher than the amount of the more active bound form, LBF.

The dose-response curve of many of these organisms to additions of such digests is admirably suited for assay of the unknown factor or factors. An example of such an assay curve is given in figure 2. Little is known of the nature of the substances supplied by these enzymatic digests, but preliminary evidence (table 2) indicates that more than a single substance is involved. Thus, of the crude supplements tested, only the enzymatic digest of casein was effective in promoting growth of *L. lactis* 104, whereas yeast extract and refined liver extract were also effective for *L. delbrueckii* 730 and *L. acidophilus* 200. Whereas glutamine gave a *partial* response with the latter organism, it was ineffective for the other two.

TABLE 2

Specificity of the response of several organisms to an enzymatic digest of casein

ADDITION TO BASAL MEDIUM*	MG PER 10 ML	INCIDENT LIGHT TRANSMITTED†		
		L. acidophilus 200	*L. delbrueckii* 730	*L. lactis* 104
None		85	98	99
Enzymatic casein digest	10	57	66	60
Enzymatic casein digest	50	57	80	46
Yeast extract (Difco)	1.0	72	82	98
Yeast extract (Difco)	10	37	61	97
Liver extract (reticulogen)	1.0	70	64	97
Liver extract (reticulogen)	10	42	42	98
L-Glutamine	0.01	76	98	99
L-Glutamine	0.1	71	96	98

* Basal medium as in table 1, but with enzymatic casein digest omitted and the acid-hydrolyzed casein replaced with the mixture of amino acid described by Henderson and Snell (1948). This medium was supplemented with 50 μg of thymidine, 3 mg ascorbic acid, 2 mμg of vitamin B_{12}, 30 μg (10 units) of LBF concentrate, and 0.02 μg of pyridoxamine phosphate when required.

† Uninoculated medium = 100. *L. acidophilus* and *L. lactis* were incubated for 42 hours, *L. delbrueckii* for 18 hours, before turbidity readings were taken.

It should be pointed out that the response of these organisms to casein digests is due to factors other than that supplied by such digests for a strain of *Lactobacillus bifidus* as reported by Tomarelli *et al.* (1949). For the latter organism, this response was evident only in the absence of thymidine, whereas in studies reported here, the growth responses were obtained in the presence of an excess of thymidine. Further work on the nature of the factors involved is planned.

Lactose. Several organisms, notably cultures of *L. bulgaricus*, initiated growth slowly in the media used. Previous work with *L. bulgaricus* Gere A (Snell, Kitay, and Hoff-Jorgensen, 1948) showed that lactose (or other β-galactosides) was required for rapid growth. The addition of lactose to the basal medium used in the present investigation speeded growth of several of the cultures, but its effect was less marked than that previously observed for *L. bulgaricus* Gere A.

SUMMARY

A survey of additional nutritive requirements of 28 cultures of lactic acid bacteria previously reported not to grow in media of known composition was made. These included cultures of *Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus delbrueckii, Lactobacillus helveticus, Lactobacillus lactis, Lactobacillus leichmannii,* and *Leuconostoc citrovorum.*

All of these cultures except *L. lactis* and *L. citrovorum* required oleic acid or other unsaturated fatty acids for growth. Eighteen of the 28 cultures required thymidine, other desoxyribosides, or vitamin B_{12} for growth. These included representatives of all of the species except *L. bulgaricus.* Only three of the organisms—one culture each of *L. acidophilus, L. delbrueckii,* and *L. helveticus*—required pyridoxamine phosphate.

Concentrates of the *L. bulgaricus* factor (LBF; Williams, Hoff-Jorgensen, and Snell, 1949) were required for the growth of 15 of these cultures, including strains of *L. acidophilus, L. bulgaricus, L. delbrueckii,* and *L. helveticus.* Evidence to show that a single unidentified factor in such concentrates was responsible for the growth effects was presented.

In addition to these pure or highly purified growth factors, one or more factors present in charcoal-treated enzymatic digests of casein are *essential* for the growth of several cultures of *L. bulgaricus, L. helveticus,* and *L. lactis,* and stimulate growth of most of the other cultures tested. Dose-response curves to such digests suitable for assay of the unknown factors involved can be obtained with several of these organisms. Differences in the specificity of the response of different organisms to different natural materials indicate that more than a single substance is involved.

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