THE MINIMAL NUTRITIONAL REQUIREMENTS OF LACTOBACILLUS BIFIDUS

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The microbiologic flora of the intestine of the breast-fed infant is characterized by the predominance of *Lactobacillus bifidus*. Investigations of the factors responsible for the establishment of this organism in the intestine of the nursling have been hampered by the lack of suitable isolation and cultivation procedures. Recently Norris *et al.* (1950) described a semisynthetic medium, supplemented with most of the factors known to stimulate the growth of lactobacilli, that will support the growth of *L. bifidus* indefinitely in the bifid phase. In the present study, the growth stimulatory activity of each of the constituents of this medium was investigated in order to determine the minimal requirements of these organisms. A knowledge of these requirements may offer an explanation of the unique predominance of *L. bifidus* in the nursling's intestine.

METHODS AND MATERIALS

The medium, developed by Norris *et al.* (1950) for the cultivation of *Lactobacillus bifidus*, is a modification of the medium described by Teply and Elvehjem (1945). The composition of the modified medium is presented in table 1. The growth promoting activity of the constituents of the medium was determined by titrimetric assay. The preparation of the inoculum and details of the assay were similar to those reported in an earlier publication (Tomarelli *et al.*, 1949). Unless stated otherwise all supplements were autoclaved with the medium.

The strains of *L. bifidus*[1] used in the present investigation were the four strains designated as Birch, Timberlain, Lockhart, and Perrish by Norris *et al.* (1950). These strains were anaerobic, possessed the characteristic bifid morphology and, as established by the present study, were identical in so far as nutritional requirements were concerned.

Tests for catalase and nitrate reduction were made by standard procedures (Wilson and Miles, 1946). The determination of volatile acids was accomplished by the Duclaux method (Kamm, 1932) and by the partition method (Delwiche, 1949).

RESULTS

Vitamin requirements. The vitamin requirements of the strains of *L. bifidus* were determined by growth experiments in which single vitamins were omitted from the otherwise complete medium of table 1. It was demonstrated that only biotin and calcium pantothenate were essential for growth of these strains

[1] We are indebted to Dr. Paul György for cultures of the strain.

(table 2). Unlike several species of *Lactobacillus* (Williams and Fieger, 1946; Williams, Broquist, and Snell, 1947), the biotin requirement could not be replaced by "tween 80" (polyoxyethylene derivative of sorbitan monooleate).

Nitrogen requirements. The amino acid nitrogen of the complete medium is supplied as an enzymatic digest of casein. Replacement of the enzymatic digest

TABLE 1

Medium (double strength concentration) for cultivation of Lactobacillus bifidus

	g		mg
K_2HPO_4	5.0	Thiamin HCl	0.4
Lactose	70.0	Riboflavin	0.4
Sodium acetate (anhyd)	50.0	Calcium pantothenate	0.8
N-Z-Case (Sheffield)	10.0	Pyridoxine HCl	2.4
Adenine, guanine, uracil, xanthine, each	0.02	Nicotinic acid	1.2
		p-Aminobenzoic acid	0.02
Alanine, cystine, tryptophan, each	0.4	Folic acid	0.02
Asparagine	0.2	Biotin	0.008

10 ml of salts B* added, pH adjusted to 6.8, diluted to 1 liter.

10 mg sterile ascorbic acid added to each 10 ml of single strength medium after sterilization.

* 10 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 0.337 g of $MnSO_4 \cdot H_2O$ in 250 ml H_2O.

TABLE 2

Vitamin requirements of strains of Lactobacillus bifidus

VITAMIN OMITTED FROM MEDIUM*	ML OF 0.1 N ACID—40 HOURS			
	Birch strain	Lockhart strain	Timberlain strain	Perrish strain
None	10.7	12.4	8.5	9.9
Thiamin HCl	10.6	13.1	8.4	9.3
Riboflavin	10.7	13.1	8.4	8.8
Nicotinic acid	10.4	12.2	8.6	9.3
Calcium pantothenate	2.0	2.2	1.6	1.1
Pyridoxine HCl	10.7	12.6	8.5	9.4
Folic acid	10.9	13.1	5.8	9.2
p-Aminobenzoic acid	10.9	13.4	8.5	9.6
Biotin	3.4	2.9	2.2	1.8

* Medium of table 1 containing acid-hydrolyzed casein (GBI—vitamin free) instead of the enzymatic digest.

with an acid hydrolysate (GBI—vitamin free) or with a mixture of crystalline amino acids did not appreciably decrease the nutritional quality of the medium, indicating that the enzymatic digest was not supplying growth factors other than the amino acids. By withdrawing one amino acid at a time from the mixture of crystalline amino acids, it was found that cysteine (cystine) was the only amino acid whose removal resulted in decreased growth. With cysteine present

in the media the additional nitrogen required for growth could be supplied by glycine, glutamine, glutamic acid, and probably by any of the other amino acids. Furthermore, an inorganic nitrogen source, i.e., ammonium acetate, was also utilized. The nitrogen of nitrates, nitrites, or of urea was not utilized (table 3).

Mineral, purine, and pyrimidine requirements. The removal of the salts B supplement from medium, simplified by limiting the vitamin and nitrogen constituents to biotin, calcium pantothenate, cysteine, and ammonium acetate, resulted in a decrease in the growth of the four strains. The effect did not appear to be specific for any one of the salts of the mixture since the individual elimination of each of the minerals of the salt B supplement did not decrease the growth of any of the strains.

The removal from the simplified medium of adenine, guanine, uracil, and xanthine, alone or in combination, did not affect the growth of the strains.

TABLE 3

Utilization of cysteine and ammonium acetate by the Birch strain of Lactobacillus bifidus

SUPPLEMENT TO NITROGEN-FREE MEDIUM* (CONCENTRATION PER ML)	ML OF 0.1 N ACID	
	40 hr	64 hr
None	0.2	0.2
2 mg ammonium acetate	0.2	0.1
0.2 mg cysteine	0.4	1.5
0.2 mg cysteine + 0.1 mg ammonium acetate	5.3	7.0
0.2 mg cysteine + 0.2 mg ammonium acetate	8.2	12.3
0.2 mg cysteine + 2.0 mg ammonium acetate	8.7	13.1
0.2 mg cysteine + 2.0 mg sodium nitrite	0.0	0.0
0.2 mg cysteine + 2.0 mg potassium nitrate	0.3	0.3
0.2 mg cysteine + 0.5 mg urea	0.7	2.8

* Buffers, sugar, salts B, biotin and calcium pantothenate.

Specific requirement for cysteine. The finding that cysteine (cystine) was the only amino acid required for the growth of the strains of *L. bifidus* led to the study of other sulfur-containing compounds. The only other compound found to possess activity was glutathione, the tripeptide with the cysteine component (table 4). The compounds tested and found to be inactive were homocysteine, homocystine, methionine, mercaptoethanol, thiopropionic acid, thiomalic acid, thioglycolic acid, sodium sulfate, sodium sulfite, sodium thiosulfate, and potassium sulfide. The combination of a sulfur-containing amino acid, methionine, and a sulfhydryl compound, homocysteine or thioglycolic acid, did not exhibit activity. The combination of serine and homocysteine was not utilized for the bacterial synthesis of cysteine (Binkley and duVigneaud, 1942).

In these experiments the various sulfur compounds were autoclaved with the medium. Since autoclaving will result in a decrease of the growth activity of cystine for a number of lactobacilli (Riesen *et al.*, 1947), a comparative study was undertaken in which aliquots of solutions of cystine and of cysteine, sterilized

by Seitz filtration, were added aseptically to the previously autoclaved medium and a second aliquot of the filtrates autoclaved with the medium. As illustrated in the data of table 4, autoclaved cystine and cysteine were more readily utilized than the supplements added aseptically.

TABLE 4

Utilization of sulfur-containing amino acids by the Birch strain of Lactobacillus bifidus

SUPPLEMENT TO THE CYSTEINE-FREE MEDIUM* (0.2 MG/ML)	ML OF 0.1 N ACID	
	40 hr	88 hr
None	0.0	0.0
Cysteine	11.9	18.8
Cysteine, aseptically	3.1	15.6
Cystine	6.9	18.0
Cystine, aseptically	3.8	13.3
Methionine	0.0	0.0
Homocysteine	0.0	0.0
Glutathione	7.3	14.7

* Buffers, sugar, ammonium acetate, salts B, biotin and calcium pantothenate.

TABLE 5

Utilization of pantethine by the Birch strain of Lactobacillus bifidus

SUPPLEMENTS TO DEFICIENT MEDIUM* (CONCENTRATION PER ML)	ML OF 0.1 N ACID	
	40 hr	88 hr
1. None	0.0	0.0
2. 0.2 mg cysteine	0.0	0.0
3. 0.4 μg calcium pantothenate	0.0	0.0
4. (2) plus (3)	11.1	19.6
5. 12 μg pantethine†	0.0	0.0
6. (2) plus 0.04 μg calcium pantothenate	13.0	19.2
7. (2) plus 0.01 μg calcium pantothenate	8.5	12.9
8. (2) plus 0.005 μg calcium pantothenate	4.9	9.0
9. (2) plus 0.04 μg pantethine	9.5	17.3
10. (2) plus 0.01 μg pantethine	7.6	13.3
11. (2) plus 0.005 μg pantethine	5.1	9.6

* Buffers, sugar, ammonium acetate, salts B, and biotin.

† Synthetic pantethine, 30,000 units/mg kindly supplied by Dr. E. E. Snell.

Utilization of pantethine by strains of L. bifidus. Chemical studies on pantethine (LBF), the growth factor for *Lactobacillus bulgaricus* (Williams, Hoff-Jørgensen, and Snell, 1949), led to identification of pantothenic acid and β-mercaptoethylamine as constituents of the pantethine molecule (Snell *et al.*, 1950). The specific requirement of the strains of *L. bifidus* for pantothenic acid and cysteine suggested that these factors were required for pantethine synthesis. However, as the data of table 5 (tubes 1 to 5) illustrate, pantethine, in an amount far in

excess of that required by lactobacilli, could not replace the activity of the combination of calcium pantothenate and cysteine. With cysteine present in the pantothenic acid-deficient medium, pantethine was found to be approximately equal to calcium pantothenate in promoting the growth of the strains of *L. bifidus*. Craig and Snell (1951) have reported similar results for a strain of *Lactobacillus casei*.

Growth of L. bifidus on minimal medium. The foregoing experiments have demonstrated that the bifid strains of *L. bifidus* will grow on a relatively simple medium consisting of lactose, sodium acetate, ammonium acetate, dipotassium phosphate, salts B, cysteine, biotin, and calcium pantothenate. The concentration of the ammonium acetate is 4 g per liter of double strength medium. The concentrations of the other ingredients of the minimal medium are the same as in the medium of table 1. (Ascorbic acid was added aseptically to each tube of medium as a routine procedure for maintenance of a favorable oxidation-reduction potential.)

The nutritional adequacy of the medium was demonstrated by the continued good growth of the strains through thirty-one daily transfers in minimal medium broth. No differences in morphology could be observed between cultures grown on minimal medium and those grown on the complete medium. Minimal medium broth cultures (40 hours' incubation) of the four strains were negative for catalase production and nitrate reduction. Approximately half of the acid produced by the organisms in minimal medium was volatile and was identified as acetic acid by both the Duclaux and partition methods.

The bifid strains used in the present study had been isolated from infant feces on agar plates of the complete medium. The simple vitamin and amino acid requirements of the bifid strains suggested that agar plates of minimal medium composition would be more selective for the isolation of strains. Preliminary to such studies it has been determined that strains of *Escherichia coli*, *Streptococcus faecalis*, *Aerobacter aerogenes*, *Proteus vulgaris*, *Lactobacillus acidophilus*, and the straight rod strains of *L. bifidus* (*parabifidus*) will not grow on agar plates of minimal medium. Each strain grew on agar plates of the complete medium.

Cultures of stools from infants and adults, and from mice and rats fed milk diets, yielded colonies on agar plates of minimal medium that consisted of gram-positive rods and was characterized by the presence of metachromatic granules. Branching of the rods was frequently observed. The number and the size of the colonies on plates of minimal medium were always smaller than those obtained with parallel plating on the complete medium. In the majority of cases inocula from colonies on plates of minimal medium did not grow when subcultured in liquid minimal medium but did grow in complete medium broth. The explanation for the latter finding is unknown at the present time.

DISCUSSION

Since its discovery by Tissier (1899) as the predominant organism of the stool of the breast-fed infant, the classification of *L. bifidus* has been a subject of controversy. Although it is the opinion of most workers that this organism is of

the genus *Lactobacillus*, various investigators have suggested *Actinomyces* (Orla-Jensen, 1943) and *Corynebacterium* (Olsen, 1949) as a more proper classification. It is probable that strain differences resulting from the variety of media and isolating procedures used by the various investigators have contributed to this uncertainty. It has been a common experience that strains of *L. bifidus* that are anaerobic (or microaerophilic) and of a bifid morphology upon primary isolation are converted upon repeated subculture to aerobic straight rods. The medium and isolation procedures of Norris *et al.* (1950) have proven successful in maintaining isolated strains of *L. bifidus* in the anaerobic bifid phase. Under these conditions the organisms produce lactic and acetic acids, are catalase negative, and do not reduce nitrates; these are reactions which advocate classification in the genus *Lactobacillus* (Norris *et al.*, 1950).

The present finding that strains of *L. bifidus* may be cultivated on a simplified medium is at variance with the generally recognized fastidiousness of the lactobacilli in regard to nutritional requirements. In addition to the better known B vitamins, representatives of this group of organisms have been shown to require pyridoxamine phosphate, vitamin B_{12}, desoxyribosides, citrovorum factor, and pantethine (Kitay and Snell, 1950). Perhaps the most striking difference in nutritional requirements of these strains in comparison to most lactobacilli is their ability to grow in a medium containing ammonium nitrogen and the single amino acid, cysteine. The requirement for cysteine appears to be specific as evidenced by the inactivity of compounds of closely related chemical structure such as homocysteine, thiopropionic acid, and β-mercaptoethylamine.

Cultures of the bifid strains in minimal medium broth, as in the complete medium (Norris *et al.*, 1950), are catalase negative, do not reduce nitrates, and produce acid of which approximately half is volatile acid. The volatile acid appears to be entirely acetic acid. Interest in the identification of the volatile acid was stimulated by close similarity of the nutritional requirements of these strains of *L. bifidus* and of the *Propionibacterium* which can also utilize ammonium nitrogen (Wood, Anderson, and Werkman, 1938) and require only the vitamins, biotin and pantothenic acid (Delwiche, 1949).

The bifid strains have been carried, since isolation two years ago, on the complete medium. It is possible that the strains have mutated to types less complex in their nutritional requirements. This is not the commonly observed mutation in which anaerobic, bifid strains of *L. bifidus* are converted to aerobic, straight rod forms. The strains used in the foregoing study will not grow in the presence of air and show a marked bifid morphology. Evidence of mutation is being sought in a current comparison of the nutritional requirements of the present strains with newly isolated strains.

SUMMARY

A study has been made of the minimal nutritional requirements of four strains of *Lactobacillus bifidus* isolated from the stools of breast-fed infants.

It was found that the strains would grow on a simplified medium comprised of lactose, buffers, minerals, ammonium salts, cysteine, and the vitamins, biotin

and calcium pantothenate. The cysteine (cystine) requirement was not replaceable by methionine, homocysteine, or related compounds. The pantothenic acid activity of pantethine was approximately equal to that of calcium pantothenate for these strains.

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