SUPPLEMENTARY GROWTH PROMOTING EFFECT OF 2-METHYL-1,4-NAPHTHOQUINONE ON *LACTOBACILLUS BIFIDUS* VAR. *PENNSYLVANICUS*

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Many investigations have been carried out concerning the ability of microorganisms to synthesize vitamin K. These investigations were stimulated by the observation that the vitamin K isolated from fish meal by Almquist and Stokstad (1935) originated from a bacterial contaminant. The subject has been summarized by Peterson and Peterson (1945) who, in comparing various microorganisms for this biosynthetic ability, have indicated the extreme variation in the amount of vitamin K produced.

In contrast to the synthetic ability, only a few species have been reported which require vitamin K for growth. Woolley and McCarter (1940) were first to demonstrate such a requirement when they observed that 2-methyl-3-hydroxy-1,4-naphthoquinone (phthiocol) would replace dried cells of *Mycobacterium phlei* in increasing the growth of *Mycobacterium johnei*. Schmidt and Büsing (1942) demonstrated a comparable effect with 2-methyl-1,4-naphthoquinone (menadione) for *Escherichia coli*. Vinet (1945) recognized the growth-promoting action of vitamin K and menadione on *Aspergillus niger*. Recently, Lev (1958) demonstrated a vitamin K requirement for a rumen strain of *Fusiformis nigrescens*.

Lactobacillus bifidus var. *pennsylvanicus*, a mutant requiring for growth human milk or more specifically, N-acetyl-D-glucosamine-containing saccharides, was isolated from feces of breast-fed infants (György *et al.*, 1952). Recently, Petuely (1957) has claimed that lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose) when added to a properly constituted milk formula will promote the growth of *L. bifidus* in the intestinal tracts of infants. Lactulose possessed no growth promoting effect *in vitro* on *L. bifidus* var. *pennsylvanicus*. We did find, however, that a preparation of crude lactulose[1] enhanced the growth of this microorganism in the presence of human milk or its active constituents and their precursors such as N-acetyl-D-glucosamine or ammonia. This supplementary effect could not be demonstrated with several other preparations of crude lactulose from the same commercial source.

This paper deals with the isolation and identification of this supplementary growth factor as 2-methyl-1,4-naphthoquinone.

[1] Bifiterlose obtained from Tervalon, Holland.

MATERIALS AND METHODS

Bacterial strains. L. bifidus var. *pennsylvanicus* (212A) is a microaerophilic mutant requiring N-acetyl-D-glucosamine-containing saccharides. *L. bifidus* strain Jackson (single cell 8; Norris *et al.*, 1950) is also microaerophilic but does not require the specific growth factor as does *L. bifidus* var. *pennsylvanicus*. *E. coli* strain B was furnished by Dr. J. S. Gots.

Culture methods. A modified Tepley and Elvehjem medium as described by György *et al.* (1954) was used to culture *L. bifidus*. When *L. bifidus* var. *pennsylvanicus* was grown in 24-L amounts, 500 ml of skimmed human milk served as the essential growth requirement. In other experiments ammonium sulfate, N-acetyllactosamine (4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine) or β-ethyl-N-acetyl-D-glucosaminide served as the essential growth requirement (György, 1957). Anaerobic conditions were accomplished with a mixture of $N:CO_2$, 9:1. The organisms were incubated for 42 hr at 37 C. *E. coli*, grown on salts-glucose medium as described by Love and Gots (1955), was incubated at 37 C with aeration for 15 hr.

Microbiological assay. The assay for the essential growth factor of *L. bifidus* var. *pennsylvanicus* was described previously (György *et al.*, 1954). This was utilized as the basis for the supplementary assay. The assays were carried out in a total volume of 10 ml. Ammonium sulfate at two levels, 1 and 3 mg, served as the

essential growth requirement. Menadione was tested, in general, at three levels, 0.01, 0.03, and 0.1 μg, for the ability to enhance each ammonium sulfate level as measured by increased acid production of the organisms. The various compounds and extracts tested in the assay were also examined over a 10-fold range corresponding to the activity of the menadione levels. In other experiments, menadione was tested over a range from 0.003 μg to 1 mg. In following the isolation of the supplementary factor from the crude lactulose preparation, 30, 90, and 300 μg of this preparation were used as a control.

Statistical evaluation. The control ammonium sulfate level of the assay and the menadione level, treated as paired samples, were analyzed for the difference in growth obtained at the two ammonium sulfate levels (1 and 3 mg) with each concentration of menadione (0.01, 0.03, 0.1, and 0.3 μg). The mean of the difference between each ammonium sulfate level and ammonium sulfate with menadione was determined. The standard deviation, standard error, and t value of this mean were calculated. The probability (P) value was derived from a t table.

Solvents. All solvents used for the isolation procedures were redistilled.

Paper chromatography. Paper chromatographic analysis was performed on Whatman paper no. 1 in descending technique. The chromatograms were irrigated in tertiary amyl alcohol:formic acid:water, 4:1:2 (solvent system I), and were developed with ammoniacal silver nitrate in the cold and 2,6-dichlorophenolindophenol and were examined under short wave, ultraviolet light. Isopropyl alcohol:acetic acid:water, 680:25:375 (solvent system II), and ethyl alcohol:acetic acid:water, 75:25:225 (solvent system III), were used as described by Green and Dam (1954). These chromatograms were examined under ultraviolet light.

Human milk. Several dialyzates of skimmed, desalted, and deproteinized human milk were prepared as described by Gauhe *et al.* (1954) and processed in a manner similar to that described for the isolation of the supplementary factor from the crude lactulose preparation.

Preparation of bacterial extracts. A 40-hr culture of *L. bifidus* strain Jackson or *L. bifidus* var. *pennsylvanicus* was used to inoculate 24 L of medium and cultured as described above. The cells were harvested by centrifugation in a Sharples supercentrifuge and washed 3 times with distilled water. The cells were autoclaved at 121.5 C, 15 psi for 45 min (Almquist *et al.*, 1938), dried *in vacuo* and refluxed with 500 ml of absolute methanol for 6 hr. The methanolic extract was freed from methanol and extracted in aqueous solution with ether for 120 hr, using 1.5 L of fresh ether every 20 hr. The extracts of each 60 hr were combined and tested in the supplementary assay.

E. coli, cultured as described above, was treated in a similar manner with the exception that the cells were not refluxed with methanol but extracted directly with ether.

Isolation of lactulose. Crude lactulose (17 g), containing 36 mg of ketose per 100 mg of fraction, determined according to Dische and Borenfreund (1951), was refluxed in 250 ml of absolute methanol for 5 hr. The supernatant was evaporated to dryness *in vacuo*. The methanolic extract (10 g), containing 48 mg of ketose per 100 mg, was dissolved in a few ml of distilled water and adsorbed on a Norite A:Celite (1:1) column (60 by 6.0 cm) prepared according to Whistler and Durso (1950). The column was washed with 21 L of distilled water until the washings gave a negative anthrone test. The column was eluted with 2.5 per cent ethanol.

Isolation of the supplementary factor. Crude lactulose (1800 g) was refluxed for 7 hr with 8 L of absolute methanol. The extract was filtered while hot and the residue washed with 1 L of methanol. The combined filtrates were evaporated to dryness *in vacuo*. The methanolic extract (966 g) was dissolved in 1 L of water and continuously extracted with ether for 12 hr. The ether extract was evaporated *in vacuo* yielding 2.97 g of a brown syrup. The latter was then chromatographed on a cellulose column (Whatman no. 3199-U; 52 by 3.2 cm in dimension) using *n*-butanol:petroleum ether (100 to 120 C):water, 38:60:2, as a solvent. The flow rate of the column was 1.6 ml per min; 5-min fractions were collected. Each tube was examined with ammoniacal silver nitrate in the cold, 2,6-dichlorophenolindophenol, and under short wave, ultraviolet light. The fractionation and the distribution of the supplementary activity is set forth in table 1. Fraction 2 was of oily consistency and partially crystallized on standing in the desiccator at room temperature. For further purification, the fraction was subject to high vacuum sublimation

TABLE 1

Fractionation of supplementary growth activity of an ether extract of the methanol soluble part of commercial lactulose†*

Fraction	Vol. of Solvent‡	Dry Wt.	Supplementary Growth Activity§	Total Units	R_f Values¶
	ml	*g*	*μg/unit*		
1	64	—	—	—	—
2	40	0.5287	1.0	5.3×10^5	0.86
					0.89
3	80	0.1524	<3	5.1×10^4	0.70
					0.78
					0.86
					0.89
4	40	0.0637	30	2.1×10^3	0.60
					0.64
					0.71
					0.78
5	160	~0.1490	10	$\sim 1.5 \times 10^4$	0.64
					0.71
					0.78
6	88	0.1190	30	4.0×10^3	0.64
					0.60
7	208	—	—	—	0.60
					0.49
					0.41

* 2.97 g of ether extract dissolved in 4 ml solvent and chromatographed on cellulose column (52 cm × 3.2 cm).

† Bifiterlose obtained from Tervalon, Holland.

‡ Solvent = *n*-butanol:petroleum ether (100–120 C):water = 38:60:2.

§ 1 unit = increased growth of *Lactobacillus bifidus* var. *pennsylvanicus* corresponding to 0.01 μg 2-methyl-1,4-naphthoquinone.

¶ Descending technique; tertiary amyl alcohol: formic acid:water = 4:1:2; developed with ammoniacal silver nitrate in the cold.

in a Willstätter tube. At 80 C and 0.1 mm pressure, 25.4 mg of pale yellow needles sublimed. These were recrystallized from 75 per cent ethanol.

RESULTS

Identification of the supplementary factor isolated from the crude lactulose preparation. The bright yellow needles formed on sublimation gave a mp 79 to 82 C (Berl, uncorrected). After one additional crystallization from benzene the mp was 101 to 102 C. The ultraviolet spectra revealed absorption at λ_{max} 246, 251, and 263 mμ which disappeared on reduction with H_2S and reappeared upon subsequent oxidation with oxygen. Analysis:

$C_{11}H_8O_2$ (172.2)

Calculated:	C 76.72	H 4.68	O 18.58
Found:	C 76.43	H 4.91	O 16.44

The molecular weight was found to be 204 (Rast).

The crystals reduced ammoniacal silver nitrate in the cold, gave a positive test with 2,6-dichlorophenolindophenol and showed quenching under ultraviolet light. The infrared spectrum was indistinguishable from authentic 2-methyl-1,4-naphthoquinone. Paper chromatographic analyses in solvent systems I, II, and III showed the crystals and 2-methyl-1,4-naphthoquinone to have identical R_f values.

Microbiological activity. The crystals, when tested in the supplementary assay as described above, were active in the amount of 0.01 μg. Identical activity was obtained with recrystallized 2-methyl-1,4-naphthoquinone (mp 109 C). Menadione increased the growth of *L. bifidus* var. *pennsylvanicus* regardless of the form of the essential growth factor, human milk, N-acetyllactosamine, β-ethyl-N-acetyl-D-glucosaminide, or ammonium sulfate. In one typical experiment, for instance, 0.09 μg of menadione gave an increased growth of 40 to 160 per cent with human milk levels of 0.04, 0.02, and 0.006 ml. Figure 1 shows how the increasing concentrations of menadione increased the growth of the organism with ammonium sulfate as the essential growth requirement under the conditions of the assay. The supplementary effect is most marked with the low levels of the essential factor. Statistical

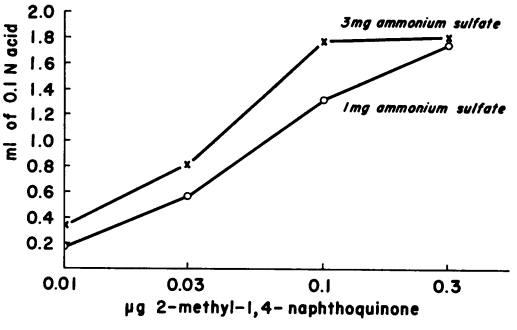

Figure 1. Increased growth of *Lactobacillus bifidus* var. *pennsylvanicus* with 2-methyl-1,4-naphthoquinone. Values are averages of 12 assays.

analysis of these growth differences represented in figure 1 showed, in all cases but one, a probability of less than 1 per cent that the particular t value obtained was due to random sampling. Menadione concentrations of 1 mg no longer increased the growth obtained with 1 and 3 mg levels of ammonium sulfate. It did not, however, inhibit the organism to a detectable extent.

The compounds tested for supplementary activity are listed in table 2. Dicumarol, 10 μg, did not inhibit the action of 0.03 and 0.1 μg of menadione or 0.3 to 3 μg of vitamin K.

In the purification of lactulose as described, fractions were obtained from the Norite A:Celite column which contained 65 to 75 mg ketose per 100 mg of fraction. These fractions had no supplementary growth promoting effect for *L. bifidus* var. *pennsylvanicus*.

Supplementary activity from human milk. The supplementary activity of skimmed human milk is difficult to assess because of the essential growth-promoting effect. When human milk was processed as described above, a fraction was obtained which possessed supplementary growth-promoting activity in 100 μg. This fraction contained no essential growth factor. Although the

TABLE 2

Supplementary growth promoting activity of various compounds for Lactobacillus bifidus var. pennsylvanicus

Compound	Supplementary activity*	Molar Ratio to Menadione
	μg/unit	
2-Methyl-1,4-naphthoquinone (menadione)	0.01	.1
2-Methyl-1,4-naphthohydroquinone	0.02	.5
2-Methyl-2,3-oxy-1,4-naphthoquinone	0.10	.1
2-Methyl-3-hydroxy-1,4-naphthoquinone (phthiocol)	Inactive to 10.0 μg	—
2-Methyl-3-phytyl-1,4-naphthoquinone (K_1)	0.30	.09
4-Amino-2-methyl-1-naphthol-hydrochloride (K_5)	0.03	.37
Tetrasodium-2-methyl-1,4-naphthohydroquinone-diphosphate (Synkayvite)	0.30	.08
2-Acetamido-3-chloro-1,4-naphthoquinone	1.00	.01
2-Acetamido-3-amino-1,4-naphthoquinone	3.00	.004
1,4-Naphthoquinone	0.03	.3
1,2-Naphthoquinone	Inactive to 3.0 μg	
Benzoquinone	Inactive to 9.5 μg	
Hydroquinone	Inactive to 30.0 μg	
Secondary isoocytyl hydroquinone	? 1.0	
9,10-Dihydroxy-9,10-diketoanthracene (anthraquinone)	Inactive to 10 μg	
Coumarin	Inactive to 3.0 μg	
Dicumarol	Inactive to 3.0 mg	
Dicumarol plus menadione	No inhibition	
Dicumarol plus vitamin K	No inhibition	
Naphth(2,3)imidazole-4,9-dione	Inactive to 100 μg	
α-Tocopherol	Inactive to 100 μg	
β-Phenylnaphtholamine	Inactive to 20 μg	
Factor 84 ($C_{16}H_{12}O_5$) antioxidant	Inactive to 5 μg	
N,N¹-diphenyl-*p*-phenylenediamine	Inactive at 10 μg	
Nordihydroguaiaretic acid	Inactive at 10 μg	
Butylated hydroxyanisole	Inactive at 10 μg	
Adenosine triphosphate	Inactive to 100 μg	
Uridine diphosphate	? at 100 μg	
Uridine diphosphate-N-acetyl-glucosamine	100	
Phosphoenolpyruvate	? 1 mg	
Pyruvic acid	Inactive at 1 mg	
Uridine	Inactive at 100 μg	
Uridylic acid	Inactive at 100 μg	

* One unit = 0.01 μg of menadione.

purification was at best 50-fold, the supplementary activity was statistically verified with probability values more significant than 5 per cent. Paper chromatographic analysis of these partially purified fractions showed in solvent system I a spot with a mobility of R_f 0.91 which gave weak quenching under ultraviolet light and in solvent system II, two spots, R_f 0.02 and 0.87 which fluoresced under ultraviolet light. These substances have not been identified.

Supplementary activity of extracts from three microorganisms. From *L. bifidus* var. *pennsylvanicus* which gives increased growth with menadione, and *L. bifidus* strain Jackson with no such requirement we obtained 10 to 11 supplementary growth-promoting units per g dry weight of cells (1 unit = the growth promoting effect of 0.01 μg of menadione). When these microorganisms were extracted directly with ether, only 1 to 3 units of supplementary substance per g dry weight of cells were found. Ether extracts of an aqueous suspension of *E. coli* strain B gave an equivalent of 50 units per g dry weight of cells.

DISCUSSION

Lactulose which was isolated from the commercial preparation and claimed to produce a flora of *L. bifidus* in infants has no essential or supplementary growth-promoting effect on *L. bifidus* var. *pennsylvanicus*. Since lactulose is not a naturally occurring constituent of human milk, its bifidogenic effect is either unrelated to the N-acetyl-D-glucosamine-containing saccharides of human milk or may be visualized as a precursor by a conversion *in situ* in the intestine to N-acetyl-lactosamine, which is a potent growth factor (Tomarelli *et al.*, 1954) for *L. bifidus* var. *pennsylvanicus*. A similar biosynthetic pathway has been indicated for the conversion of fructose-6-phosphate to N-acetyl-D-glucosamine-6-phosphate by Leloir and Cardini (1956).

The enhancing effect present in the one preparation of commercial lactulose led to the identification of 2-methyl-1,4-naphthoquinone as a supplementary factor for *L. bifidus* var. *pennsylvanicus*. The origin of menadione in this commercial lactulose and its role in the metabolism of *L. bifidus* var. *pennsylvanicus* are at present obscure. Vitamin K has been demonstrated to take part in the electron transport chain by Martius and Nitz-Litzow (1953) using livers from vitamin K-deficient chicks, by Arnon *et al.* (1958) in systems of isolated chloroplasts, and by Brodie *et al.* (1957) with extracts of *M. phlei*. It is possible that vitamin K acts in the energy yielding system of microaerophilic *L. bifidus* var. *pennsylvanicus*. Dicumarol does not, however, reverse the action of menadione on the intact organism under our experimental conditions. It is attractive to speculate that the 1,4-naphthoquinone nucleus might have a coenzyme function in the phosphorylation of amino sugars. It should be, however, borne in mind that the supplementary effect of menadione and related substances is more consistently demonstrable with suboptimal amounts of the essential growth factor present in human milk or of its precursors.

Guérillot-Vinet (1948) points out that high concentrations of menadione will inhibit the organisms for which it acts as a growth-promoting agent. In contrast, *L. bifidus* var. *pennsylvanicus* is not inhibited by levels of menadione as high as 1 mg. At this level, however, there is no supplementary effect under the conditions of the assay.

Human milk exhibits a supplementary effect, the principle of which has not as yet been identified. Although skimmed human milk was used exclusively, it is conceivable that trace amounts of vitamin K may be responsible for this effect.

The extracts from *L. bifidus* strain Jackson and *L. bifidus* var. *pennsylvanicus* were studied because of their differences in requirements for essential and supplementary growth factors. It was thought that the former strain might synthesize its own K vitamin in readily demonstrable amounts. Under our experimental conditions, however, this was not the case, as extracts of both strains exhibited approximately the same supplementary activity.

Studies on the role of vitamin K in cellular metabolism have been hampered to some extent by the lack of a satisfactory method for analysis of vitamin K and related compounds. The possibility that *L. bifidus* var. *pennsylvanicus* can be used for a microbiological assay is to be considered. With the limited number of compounds tested, the organism appears selective for 1,4-naphthoquinone and derivatives with the exception of phthiocol. In fact, as shown in table 2, any substitution on the third carbon reduces the activity. It was conceivable that any antioxidant could enhance the growth of this organism. How-

ever, not any of these substances tested, including α-tocopherol, had microbiological activity under the conditions of the supplementary assay.

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SUMMARY

A supplementary growth factor for *Lactobacillus bifidus* var. *pennsylvanicus*, a mutant requiring N-acetyl-D-glucosamine-containing saccharides for growth, was isolated in crystalline form from a commercial preparation of crude lactulose. On the basis of the ultraviolet absorption spectrum, infrared spectrum, elementary analysis, and chromatographic behavior, the crystals were identified as 2-methyl-1,4-naphthoquinone (menadione). Menadione, in concentrations of 0.001 μg per ml of medium, increased the growth of this organism 100 to 200 per cent in the presence of lower levels of the essential growth factor.

Of the compounds examined the supplementary growth effect was limited to menadione, vitamin K_1, the commercially available vitamins, K_5 and Synkayvite, and 1,4-naphthoquinone. Phthiocol, benzoquinone, 1,2-naphthoquinone, and the antioxidants tested were inactive. Dicumarol did not reverse the activity of menadione or vitamin K under our experimental conditions.

Preliminary attempts were made to isolate the supplementary factor present in ether extracts of skimmed human milk.

A supplementary growth-promoting effect was demonstrated in ether extracts of *L. bifidus* strain Jackson, *L. bifidus* var. *pennsylvanicus*, and *Escherichia coli* strain B.

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