DESOXYRIBOSIDES AND VITAMIN B_{12} AS GROWTH FACTORS FOR LACTIC ACID BACTERIA[1]

ESTELLE KITAY, WALTER S. McNUTT, AND ESMOND E. SNELL

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

Received for publication February 27, 1950

Rogosa, Tittsler, and Geib (1947) reported that many strains of *Lactobacillus bulgaricus*, *Lactobacillus lactis*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, and *Lactobacillus leichmannii* failed to grow in semisynthetic media similar to those used for vitamin assay with other lactic acid bacteria, even though all of the then known B vitamins were added. Since that time oleic acid (Williams, Broquist, and Snell, 1947; Hutchings and Boggiano, 1947), pyridoxamine phosphate (McNutt and Snell, 1948, 1950), and an unidentified substance provisionally termed "LBF" (*Lactobacillus bulgaricus* factor, Williams, Hoff-Jørgensen, and Snell, 1949) have been shown to be essential for the growth of certain of these organisms. A series of preliminary reports (e.g., Shorb, 1948; Kitay, McNutt, and Snell, 1949; Kocher and Schindler, 1949; Hoff-Jørgensen, 1949) has also established that certain of these organisms require vitamin B_{12}, desoxyribosides, or certain reducing agents for growth.

This report represents part of an investigation to determine the hitherto unidentified growth factors required by the above-named organisms. It describes the specificity and variability of several lactic acid bacteria in their requirement for desoxyribosides, vitamin B_{12}, or reducing agents, and gives the experimental conditions under which these were determined.

EXPERIMENTAL METHODS AND RESULTS

Stock cultures. The various organisms tested were transferred biweekly in litmus milk supplemented with 0.5 per cent glucose and 0.5 per cent Difco yeast extract. Following transfer, the cultures were incubated at 37 C until the milk coagulated (16 to 24 hours) and were then stored at 10 C for the remainder of the 2 weeks.

Inoculum medium. Each tube of inoculum medium contained 5 ml of the appropriate basal medium (see below), an enzymatic digest of desoxyribonucleic acid equivalent in growth-promoting activity to 50 μg of thymidine,[2] 1 ml of

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Division of Research Grants and Fellowships, National Institutes of Health, and Eli Lilly and Company, Indianapolis, Indiana. We are indebted to Dr. T. G. Brady, Dr. O. L. Lampen, Dr. E. Hoff-Jørgensen, and to the P. A. Levene collection for small samples of some of the desoxyribosides. Subsequently, certain of these were prepared from desoxyribonucleic acid as described elsewhere (McNutt and Snell, 1950). Dr. T. H. Jukes kindly supplied samples of vitamin B_{12b} and of the *L. citrovorum* factor.

[2] This digest was the $MgSO_4$ concentrate of enzymatically hydrolyzed thymonucleic acid prepared by the procedure described by Klein (1938).

TABLE 1

Composition of the basal medium

COMPONENT	AMOUNT PER 100 ML DOUBLE-STRENGTH MEDIUM	COMPONENT	AMOUNT PER 100 ML DOUBLE-STRENGTH MEDIUM
	mg		*μg*
Glucose	2,000	Calcium pantothenate	80
Acid-hydrolyzed casein*	1,000	Nicotinic acid	80
Enzymatic casein digest†	400	Riboflavin	80
Asparagine	20	Thiamine	40
DL-Tryptophan	20	*p*-Aminobenzoic acid	40
L-Cystine	40	Pyridoxal hydrochloride	40
Adenine hydrochloride	2	Folic acid	2
Guanine	2	Biotin	0.4
Uracil	2		
Oleic acid	2		
Sorbitan monooleate	200	Salts	See table 2

* Labco "vitamin-free" casein (300 g) is refluxed at least 12 hours with 3 liters of 20 per cent HCl. It is evaporated under vacuum to a thick syrup, then diluted to 3 liters with distilled water. This concentration procedure is repeated twice. The final concentrate is diluted to 1 liter. The pH is adjusted to 2.5 with KOH. The mixture is stirred with 30 g of activated carbon (Darco G 60) for 15 minutes, then filtered with the aid of "filter-cel." The pH is adjusted to 6.8 with KOH after the volume of solution is brought to 3 liters and the charcoal treatment and filtration are repeated. One ml of the solution contains the equivalent of 100 mg of original casein.

† Labco "vitamin-free" casein (120 g) is suspended by gradual addition in 2 liters of 0.8 per cent sodium bicarbonate solution. One g of pancreatin, suspended in 15 to 20 ml of water is added to this uniform suspension, and the pH of the mixture is adjusted to 8.0 with KOH. The mixture is covered with a thin layer of toluene, shaken well, and allowed to stand for 56 hours at 37 C. At the end of this period the mixture is steamed for 20 to 30 minutes and cooled to room temperature. The pH of the solution is adjusted to 6.8 with glacial acetic acid and filtered with mild suction. Sixty g of activated carbon (Darco G 60) are added to the filtrate; the mixture is stirred for 30 minutes, then filtered with suction. This filtrate is adjusted to pH 3.0 with glacial acetic acid and HCl, stirred for 30 minutes with 24 g of activated carbon, and filtered with the aid of filter-cel. A total of 80 ml of glacial acetic acid is used. The final clear filtrate is diluted to 2.4 liters and contains 40 to 50 mg of solids per ml.

TABLE 2

Salt concentrations of the various media

COMPONENT	GRAMS PER 100 ML OF DOUBLE-STRENGTH MEDIUM			
	Medium 1	Medium 2	Medium 3	Medium 4
Sodium acetate*	2.0	2.0	0.20	3.2
Sodium citrate	2.0	—	2.0	—
KH_2PO_4	0.60	0.10	0.60	0.12
K_2HPO_4	0.60	0.10	0.60	0.12
$MgSO_4 \cdot 7H_2O$	0.56	0.16	0.56	0.04
$MnSO_4 \cdot H_2O$	0.084	0.024	0.084	0.004
$FeSO_4 \cdot 7H_2O$	0.028	0.004	0.028	0.002
NaCl	0.008	0.004	0.008	0.002

* The amounts of sodium acetate indicated are in addition to that supplied by the enzymatic casein digest.

filtered tomato juice,[3] and water to make 10 ml. Tubes were autoclaved for 8 minutes at 15 pounds pressure and stored until needed.[4]

Inoculum. Two loopfuls of the milk culture were transferred to a tube of the inoculum medium, which was incubated for 18 to 24 hours at 37 C. The cells were centrifuged, washed twice, then resuspended in 10 ml of 0.9 per cent NaCl solution. One drop of this heavily turbid suspension was used to inoculate each experimental tube of 10 ml.

Basal medium. The composition of that portion of the basal medium common to all media used is shown in table 1. The salt mixtures used to complete various individual media are shown in table 2. The double-strength media were prepared as needed for assay purposes from stock solutions of salts, vitamins, etc., and the pH was adjusted to 6.6 with KOH.

Procedure. Materials to be tested were dispensed in a series of uniform 18-by-150-mm pyrex test tubes, water was added to 5 ml, and then 5 ml of the basal medium, appropriately supplemented as noted in the tables, were added. The tubes were covered with aluminum caps, autoclaved at 120 C for 5 to 7 minutes, cooled, inoculated, shaken to disperse the organisms throughout the medium, and incubated at 37 C. Growth was estimated photometrically, directly in the selected tubes, with an Evelyn colorimeter and the 660-mμ filter. Turbidities were measured at intervals during a period of 18 to 72 hours after inoculation.

When unheated materials were tested, solutions were neutralized, sterilized by filtration through sintered glass or Seitz filters, and added aseptically to assay tubes after the medium had been autoclaved and cooled.

Growth response of the various organisms to thymidine. As previously reported (Snell, Kitay, and McNutt, 1948), addition of thymidine to a medium similar to those of table 1 permitted growth of two strains of *Lactobacillus leichmannii* and one of *Leuconostoc citrovorum.* In all, 25 additional strains of lactic acid bacteria, obtained from Dr. R. P. Tittsler, Dr. W. B. Sarles, and the American Type Culture Collection and reported not to grow on a chemically defined medium containing all of the known nutrients, were tested for response to thymidine. For this screening assay, organisms were inoculated into medium 1 supplemented with 1 ml of filtered tomato juice, 30 μg of an LBF concentrate (Williams, Hoff-Jørgensen, and Snell, 1949) and 0.02 μg of pyridoxamine phosphate per 10 ml of medium and into this same medium with 50 μg of added thymidine per 10

[3] The tomato juice was prepared by boiling 350 g of fresh tomatoes with 1 liter of distilled water for 30 minutes. The preparation was filtered and stored at −4 C. Later in the investigation, when factors supplied by the tomato juice became known (Kitay and Snell, 1950), this supplement was replaced in some media by small amounts of pyridoxamine phosphate and a concentrate of the *Lactobacillus bulgaricus* factor (LBF; Williams, Hoff-Jørgensen, and Snell, 1949) as indicated in the tables. The latter concentrate was purified about 1,000 times over yeast extract by an extension of published procedures and was free of desoxyribosides and vitamin B_{12}.

[4] For *L. acidophilus* S and *L. helveticus* S, an enriched inoculum medium consisting of 10 ml of the basal medium, 50 mg of Difco proteose peptone, 16 mg of Difco yeast extract, and 8 mg of malt extract was used since use of a semisynthetic medium resulted in growth in the unsupplemented basal medium on subsequent trials.

ml. Of the 28 strains tested, 10 grew on the basal medium but 18 grew well only on the medium supplemented with thymidine (table 3) and showed little or no growth in the medium without thymidine. Subsequent experiments showed

TABLE 3

Growth response of 18 strains of lactic acid bacteria to thymidine

ORGANISM*	ATCC NO.	ADDITIONS TO BASAL MEDIUM†		MEDIUM USED FOR SUBSEQUENT STUDY
		None	Thymidine (50 μg/10 ml)	
		% incident light transmitted‡		
Lactobacillus acidophilus 200	—	94	67	2
Lactobacillus acidophilus 203	314	94	61	2
Lactobacillus acidophilus 204	332	91	37§	2
Lactobacillus acidophilus 206	4357	99	48	2
Lactobacillus acidophilus 207	4962	95	60	2
Lactobacillus acidophilus 213	4355	98	30	2
Lactobacillus acidophilus	832	94	42	2
Lactobacillus acidophilus	4356	99	58	2
Lactobacillus acidophilus S	—	94	42	1
Leuconostoc citrovorum	8081	97	38§	4
Lactobacillus delbrueckii 730	9649	99	50	2
Lactobacillus delbrueckii	4796	99	48	2
Lactobacillus delbrueckii	4913	94	58	2
Lactobacillus helveticus S	—	96	40	1
Lactobacillus lactis 104	—	98	41§	4
Lactobacillus leichmannii 313	7830	99	50	3
Lactobacillus leichmannii 326	4797	93	63	3
Lactobacillus leichmannii 327	7831	92	41	3

* The strain numbers following the culture name are the designations in Dr. Tittsler's collection; those in column 2, of the American Type Culture Collection. The letter "S" indicates that the culture was obtained from Dr. W. B. Sarles, Department of Agricultural Bacteriology, University of Wisconsin.

Cultures that grew on the basal medium used (see below) were *L. acidophilus* 217 (4857), *L. bulgaricus* 2 (525), *L. bulgaricus* 10 (8018), *L. bulgaricus* 14 (- - - -), *L. bulgaricus* - - (7995), *L. bulgaricus* - - (8001), *L. gayonii* V-616 (8289), *L. helveticus* 77 (- - - -), *L. helveticus* 80 (- - - -), and *L. lactis* 108 (- - - -).

† Medium 1 (tables 1 and 2) plus 1 ml filtered tomato juice, 30 μg of an LBF concentrate, and 0.02 μg of pyridoxamine phosphate per 10 ml.

‡ Uninoculated medium = 100. Unless otherwise specified, incubation was for 24 hours at 37 C. No growth without thymidine occurred even though incubation was continued for 3 days.

§ Incubated 44 to 51 hours at 37 C.

that better growth was obtained without citrate with some of these organisms and with lower total salt concentration with others. The media subsequently used for the various organisms are indicated in column 5 of table 3.

Nonspecificity of thymidine as a growth factor for these organisms. Preliminary reports (Kitay, McNutt, and Snell, 1949; Hoff-Jørgensen, 1949; Kocher and

Schindler, 1949) established that desoxyribosides other than thymidine were as effective as the latter in promoting growth of some lactic acid bacteria, and that for some organisms vitamin B_{12} had an equivalent growth-promoting effect (cf. also Shive *et al.*, 1948; Hoffmann *et al.* 1948; and Skeggs *et al.*, 1948). For the majority of the "thymidine-requiring" organisms listed in table 3, thymidine, hypoxanthine desoxyriboside, adenine desoxyriboside, cytosine desoxyriboside, and guanine desoxyriboside showed equal activity in promoting growth. Behavior of such organisms is typified by that of *L. leichmannii* 313 and *L. acidophilus* 832, shown in table 4. Some of the organisms, however, showed distinct preference for one or another of the desoxyribosides. *L. delbrueckii* 730, for example, grew heavily within 24 hours in media containing thymidine, and did not utilize other desoxyribosides significantly within this time. At 48 hours, other desoxyribosides also showed some activity, but not so great as thymidine (table 4). Similar behavior, not tabulated, was shown by *L. acidophilus* 204, 206, 207, and 4356. In a few cases (e.g., *L. acidophilus* 200, 203, 213, and S), cytosine desoxyriboside stimulated more rapid and heavier growth than any of the other desoxyribosides. High levels of thymonucleic acid, prepared by Hammarsten's procedure (1924), promoted growth of most of these organisms in a longer period of time (table 4).

Vitamin B_{12} replaced desoxyribosides for most of these organisms, as illustrated by the behavior of *L. leichmannii* 313 and *L. acidophilus* 832 (table 4). However, *L. delbrueckii* 730 and *L. acidophilus* 204 showed no response to this vitamin at levels as high as 50 to 100 mμg per 10 ml of medium. For these organisms, thymidine or, less efficiently, other desoxyribosides and desoxyribonucleic acid must be considered as essential growth factors under these conditions. Thymine, high levels of folic acid (up to 5 μg per 10 ml), cobaltous ion, and ribosides such as adenosine and inosine were ineffective in promoting growth of any of these organisms. A hydrolyzate of hypoxanthine desoxyriboside, prepared in a manner reported to yield the theoretical quantity of D-2-desoxyribose (Levene and Bass, 1931), was also ineffective, as was a synthetic preparation of this sugar kindly supplied by Dr. H. A. Lardy.

Ascorbic acid and other reducing agents. Several reports have shown that ascorbic acid, anaerobiosis, or prolonged periods of autoclaving will permit growth of organisms such as *L. lactis* or *L. leichmannii* in the absence of vitamin B_{12} or desoxyribosides, which are otherwise required (Kitay, McNutt, and Snell, 1949; Shive, Ravel, and Eakin, 1948; Kocher, 1949; Greene, Brook, and McCormack, 1949). Koditschek, Hendlin, and Woodruff (1949) showed with *L. lactis* that these effects resulted from a lowered oxidation-reduction potential in the medium, and that the requirement for vitamin B_{12} was enhanced by aeration or by oxidizing agents. In this investigation similar results were encountered. Ascorbic acid or other reducing agents such as sodium thioglycolate, cysteine, glutathione, or alkali-treated glucose were found to promote the growth of many, but not all, of the organisms studied. Representative data, which compare the growth effects of ascorbic acid, thymidine, and vitamin B_{12}, are given in table 5. The figures given were selected at incubation times when the growth effect of each

compound had reached a maximum. For some organisms (e.g., *L. lactis* 104, *L. acidophilus* S) ascorbic acid, thymidine, and vitamin B_{12} had equivalent effects. For many others (e.g., *L. leichmannii* 313, *L. delbrueckii* 4796) thymidine

TABLE 4

Comparative growth-promoting activity of desoxyribosides and vitamin B_{12} for several organisms

ADDITIONS TO BASAL MEDIUM†	AMOUNT PER 10 ML	PER CENT OF INCIDENT LIGHT TRANSMITTED*							
		L. leichmannii 313		*L. acidophilus* 832		*L. delbrueckii* 730		*L. acidophilus* 200	
		19 hr	48 hr	22 hr	101 hr	22 hr	48 hr	23 hr	72 hr
None	0	95	95	93	90	95	95	94	93
Thymine desoxyriboside	2 μg	82	66	82	78	92	92	76	60
	5	77	48	74	69	79	80	73	56
	10	72	39	65	56	60	57	70	50
	50	66	37	55	55	55	46	63	50
Adenine desoxyriboside	2 μg	82	67	84	79	94	91		
	5	77	49	73	71	96	86		
	10	73	39	68	58	89	78		
	50	65	36	56	54	90	64	69	49
Cytosine desoxyriboside	2 μg	82	67	83	78	97	92		
	5	79	49	75	70	93	89		
	10	73	40	67	57	91	84		
	50	64	37	55	53	89	68	42	49
Guanine desoxyriboside	2 μg	82	66	82	77	95	97		
	5	76	48	73	70	94	88		
	10	70	40	65	56	90	75		
	50	58	36	54	54	90	63	67	51
Hypoxanthine desoxyriboside	2 μg	82	67	85	78	95	92		
	5	79	49	75	70	94	83		
	10	73	39	68	57	92	70		
	50	66	37	56	53	89	61	60	50
Desoxyribonucleic acid	20 μg	95	72	94	79	69	63		
	50	89	61	94	61	61	55	94	85
	100	81	59	93	55	57	50		
	500	71	43	89	57	59	50	90	53
Vitamin B_{12}	1 mμg	83	70	95	95	95	95	88	86
	5	55	43	88	80	96	95		
	10	37	31	78	72	96	96		
	50	30	25	56	53	95	95		

* Uninoculated medium = 100.

† Basal medium is that indicated in the fourth column of table 1 plus 1.0 ml filtered tomato juice per 10 ml.

and vitamin B_{12} gave closely similar effects, but ascorbic acid was much less effective, both in the maximum cell yields achieved and in the rate at which these were produced. For *L. acidophilus* 204, ascorbic acid permitted growth that was frequently heavier and always more rapid than that induced by thymidine. It will be noted that vitamin B_{12} was inactive for this organism under

the conditions used here. Finally, organisms such as *L. delbrueckii* 730 exist for which neither vitamin B_{12} nor ascorbic acid is effective in replacing thymidine. Similar data, with some additional organisms and at different levels of supplementation, are shown in table 6.

Relation of the enzymatic casein digest of the medium to the growth response to ascorbic acid. Welch and Wilson (1949) observed that ascorbic acid replaced vitamin B_{12} for *L. leichmannii* only in media that contained enzymatic casein digests, and that the effectiveness of ascorbic acid was much enhanced by autoclaving it with the medium. On these grounds, they suggest that oxidation prod-

TABLE 5

Comparative effects of thymidine, vitamin B_{12}, and ascorbic acid in promoting growth of lactic acid bacteria

ORGANISM	INCUBATION TIME	ADDITIONS PER 10 ML OF BASAL MEDIUM*			
		None	Thymidine, 50 μg	Vitamin B_{12}, 1 mμg	Ascorbic acid, 3 mg
		Per cent of incident light transmitted†			
	hr				
L. acidophilus 200	51	94	57	91	78
L. acidophilus 204	51	92	38	92‡	41
L. acidophilus 206	72	93	31	92	83
L. acidophilus 832	26	95	44	78‡	89
L. acidophilus S	19	88	35	43	40
L. delbrueckii 730	26	100	39	99	99
L. delbrueckii 4796	23	99	48	44	80
L. helveticus S	19	78	53	55	52
L. lactis 104	44	95	41	44	47
L. leichmannii 313	26	94	43	37‡	78

* Basal media as indicated in table 3, supplemented with 30 μg of an LBF concentrate and 0.02 μg of pyridoxamine phosphate per 10 ml for those organisms that require these factors (McNutt and Snell, 1948, 1950; Kitay and Snell, 1950).

† Uninoculated medium = 100.

‡ Ten mμg of vitamin B_{12} were added to these cultures.

ucts of vitamin B_{12} remain in the enzymatic digests of casein even after charcoal treatment, and that reducing agents promote growth only because they reduce such products to vitamin B_{12}. We have obtained similar data with *L. leichmannii* and many other organisms; certain exceptional cases, however, lead us to question the interpretation of the results offered by Welch and Wilson. Ten of 14 organisms that responded to ascorbic acid did so only when the latter was autoclaved with the medium. *L. acidophilus* 204, *L. lactis* 104, *L. helveticus* S, and *L. acidophilus* S, however, grew equally well or better when ascorbic acid (or thioglycolic acid, glutathione, or cysteine) was added aseptically to a previously autoclaved medium. Similarly, although most of the organisms that responded to ascorbic acid did so only when an enzymatic casein digest was present in the medium (e.g., *L. leichmannii* 313), three organisms (*L. acidophilus* 204, *L. aci-*

dophilus S, and *L. helveticus* S) responded equally well in the absence of this digest. Illustrative data are given in table 6.

These data and those of table 5 demonstrate that ascorbic acid cannot be acting solely through the production of vitamin B_{12} from oxidized products present in the enzymatic digest of casein since (a) it is active for several organisms in the absence of such digests, and (b) it promotes growth of certain organisms that cannot utilize vitamin B_{12} under the same conditions. It also seems apparent that the effect is not due to the production of utilizable forms of the

TABLE 6

Comparative response of several bacteria to ascorbic acid, thymidine, and vitamin B_{12} in the presence and absence of an enzymatic casein digest

ORGANISM	INCUBATION TIME	BASAL MEDIUM*	ADDITIONS PER 10 ML OF BASAL MEDIUM			
			None	Vitamin B_{12}, 5 mμg	Thymidine, 50 μg	Ascorbic acid, 5 mg
			Per cent of incident light transmitted†			
	hr					
L. acidophilus 204	25	AA	100	100	72	56
		ECD	100	100	59	38
L. acidophilus 206	92	AA	100	100	57	100
		ECD	100	100	45	84
L. acidophilus 213	45	AA	100	63	64	100
		ECD	100	60	54	65
L. acidophilus S	72	AA	100	45	50	40
		ECD	93	35	44	37
L. delbrueckii 4913	45	AA	100	75	70	100
		ECD	100	77	69	70
L. helveticus S	72	AA	100	42	49	38
		ECD	96	40	42	35
L. leichmannii 313	92	AA	100	40	42	100
		ECD	100	44	35	70

* ECD = basal medium as indicated in table 3 supplemented with 30 μg of an LBF concentrate and 0.02 μg of pyridoxamine phosphate per 10 ml when required. This medium contains enzymatic digest of casein. AA = basal medium as above, except both the enzymatic digest of casein and the acid hydrolyzate of casein were replaced by the mixture of purified amino acids described by Henderson and Snell (1948).

† Uninoculated medium = 100.

desoxyribosides. The nature of the action of ascorbic acid and of its interaction with enzymatic casein digests in promoting growth of many of these organisms thus remain unexplained.

Variation in the quantitative requirement for vitamin B_{12}. All the organisms studied above have responded to thymidine (or to other active desoxyribosides) over the same concentration range. In marked contrast, the amount of vitamin B_{12} necessary to elicit a growth response varies markedly from one organism to another. This is illustrated by figure 1. *L. acidophilus* 213 requires about five times as much vitamin B_{12} for growth as does *L. leichmannii* 313. The reason for such large differences in the magnitude of the requirement for vitamin B_{12} is

not known; such differences may reflect differences in cell permeability to the large vitamin B_{12} molecule, differences in the efficiency of utilization, or differences in the rate of destruction. When autoclaved with the medium, vitamin B_{12} is roughly seven times as active as vitamin B_{12b} (Pierce *et al.*, 1949) for both *L. acidophilus* 213 and *L. leichmannii* 313 (figure 1). Separate experiments showed both compounds to have equal growth-promoting activity following aseptic addition to previously autoclaved media.

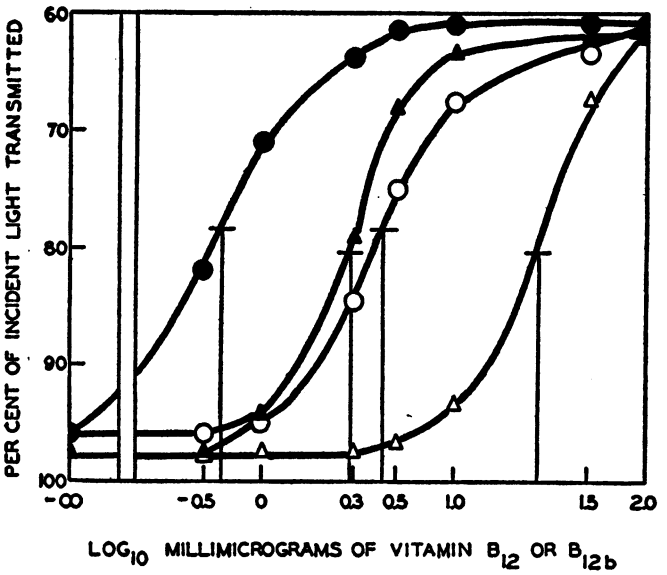

Figure 1. Comparative activities of vitamins B_{12} and B_{12b} in promoting growth of *L. acidophilus* 213 and *L. leichmannii* 313. ●, *L. leichmannii*, vitamin B_{12}; ○, *L. leichmannii*, vitamin B_{12b}. ▲, *L. acidophilus*, vitamin B_{12}; △, *L. acidophilus*, vitamin B_{12b}. Turbidity measurements were made after 20 hours of incubation, basal media as in table 6. Both vitamins were autoclaved with the culture medium.

The response of L. citrovorum to thymidine. In contrast to the other organisms tested, *L. citrovorum*, although it responded to thymidine (table 3), did not respond to additions of any of the other four desoxyribosides, to desoxyribonucleic acid, to ascorbic acid, or to vitamin B_{12}. It differed from *L. delbrueckii* 730, which showed somewhat similar behavior (table 4), in that reticulogen (a refined liver extract) was highly active in promoting its growth. Others (Sauberlich and Baumann, 1948, 1949) have shown that a growth factor of unknown structure but apparently closely related to folic acid (Sauberlich, 1949; Bond *et al.*, 1949) is highly active in replacing thymidine in the nutrition of this organism. Concentrates of this factor were ineffective in replacing desoxyribosides for any of the other organisms tested.

DISCUSSION

No single explanation for the observed interchangeability of desoxyribosides, vitamin B_{12}, and reducing agents in the nutrition of many of these organisms is

apparent. It was previously suggested (Kitay, McNutt, and Snell, 1948) that vitamin B_{12} might be an essential catalyst (e.g., a coenzyme) in the formation of desoxyribosides. When the products of its catalytic action (i.e., desoxyribosides) were supplied to the cells preformed, the catalyst might no longer be required. No experimental evidence bearing directly upon this hypothesis has appeared. It adequately accounts for the existence of organisms such as *L. delbrueckii* 730, which require thymidine even though vitamin B_{12} is present, since such organisms may well have lost certain apoenzymes essential for the synthesis of thymidine. The postulate of Welch and Wilson (1949), according to which reducing agents function by reactivating inactive oxidized forms of vitamin B_{12} furnished with enzymatic digests of casein, would permit an explanation of their action fully in line with the hypothesis above. However, although this may explain the action of reducing agents in promoting the growth of some organisms, it is insufficient as an explanation for others, as pointed out elsewhere in this report. Another possible explanation (McNutt and Snell, 1950) is that certain organisms synthesize vitamin B_{12} (or its physiological equivalent) below a given critical oxidation-reduction potential and consequently grow in its absence. Even this postulate, however, fails to explain the equivalence of ascorbic acid and desoxyribosides for organisms such as *L. acidophilus* 204, for which vitamin B_{12} was inactive under the conditions used above. It may be that such organisms cannot utilize external supplies of vitamin B_{12} but can utilize that which they synthesize. Some evidence for variations in cellular permeability to vitamin B_{12} is provided by the markedly different magnitude of the requirement for this vitamin exhibited by different organisms. The complexity of the vitamin B_{12} molecule and the occurrence of both vitamin B_{12} and vitamin B_{12b} in nature suggest that other forms of the vitamin may also occur. It is quite possible that the combined effects of ascorbic acid and enzymatic protein digests, though demonstrably not due to the production of vitamin B_{12} per se, may be due to fragments of this vitamin which are active for a wider range of organisms than is vitamin B_{12} itself. Additional experimentation is required to assess the credibility of these and several other possible explanations for the observed interrelationships.

The existence of organisms showing the markedly different specificities toward desoxyribosides, vitamin B_{12}, and reducing agents described in this paper should be of considerable value in permitting the development of more specific assay methods for the vitamin, in the detection of possible additional forms in which it occurs, and in the elucidation of its mode of action.

SUMMARY

Eighteen strains of lactic acid bacteria, representative of six different species and hitherto not cultured in media of known composition, were examined for certain additional nutritional requirements.

None of these bacteria grew in a medium complete with respect to known amino acids and synthetic vitamins and supplemented with tomato juice and an enzymatic digest of casein. All grew when thymidine was added to this medium.

In most cases, thymidine could be replaced by either hypoxanthine desoxyriboside, adenine desoxyriboside, guanine desoxyriboside, cytosine desoxyriboside, or high levels of desoxyribonucleic acid. Individual differences in the availability of the various desoxyribosides were noted. Several organisms (e.g., *Lactobacillus delbrueckii* 730) grew more rapidly and heavily with thymidine than with other desoxyribosides, and one, *Leuconostoc citrovorum* 8081, grew only with thymidine. For a few other organisms, cytosine desoxyriboside appeared to be more active than other desoxyribosides. Most organisms showed delayed growth with desoxyribonucleic acid.

Vitamin B_{12} replaced thymidine (or other desoxyribosides) for many, but not all, of these organisms. *L. delbrueckii* 730 and *Lactobacillus acidophilus* 204 are examples of organisms that respond to thymidine, but not to vitamin B_{12}.

Ascorbic acid, thioglycolic acid, cysteine, or glutathione replaced thymidine, other desoxyribosides, or vitamin B_{12} for many of these organisms in media that contained an enzymatic digest of casein. For most organisms, these reducing agents were ineffective in eliminating the requirement for these growth factors when the enzymatic casein digest was omitted from the medium. *L. acidophilus* 204, *L. acidophilus* S, and *Lactobacillus helveticus* S, however, grew well when ascorbic acid was supplied under these latter conditions.

When added aseptically to previously autoclaved media, vitamin B_{12} and vitamin B_{12b} were equally active in promoting growth. Sterilization with the medium reduced the activity of vitamin B_{12b} to approximately one-seventh that of vitamin B_{12}. Although all organisms required similar amounts of the active desoxyribosides for growth, a very considerable variation in the amounts of vitamin B_{12} required was observed.

REFERENCES

Bond, T. J., Bardos, T. J., Humphreys, J., and Shive, W. 1949 Relationship of the folinic acid group and the *Leuconostoc citrovorum* factors. J. Am. Chem. Soc., **71**, 3852.

Greene, R. D., Brook, A. J., and McCormack, R. B. 1949 Some conditions which affect the assay of vitamin B_{12} with *Lactobacillus lactis* Dorner. J. Biol. Chem., **178**, 999–1000.

Hammarsten, O. 1924 Zur Kenntnis der biologischen Bedeutung der Nucleinsäureverbindungen. Biochem. Z., **144**, 383–466.

Henderson, L. M., and Snell, E. E. 1948 A uniform medium for determination of amino acids with various microorganisms. J. Biol. Chem., **172**, 15–29.

Hoff-Jørgensen, E. 1949 Difference in growth-promoting effect of desoxyribosides and vitamin B_{12} on three strains of lactic acid bacteria. J. Biol. Chem., **178**, 525–526.

Hoffman, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H. 1948 Response of *Lactobacillus leichmannii* 313 to the antipernicious anemia factor. J. Biol. Chem., **176**, 1465–1466.

Hutchings, B. L., and Boggiano, E. 1947 Oleic acid as a growth factor for various lactobacilli. J. Biol. Chem., **169**, 229–230.

Kitay, E., McNutt, W. S., and Snell, E. E. 1949 The non-specificity of thymidine as a growth factor for lactic acid bacteria. J. Biol. Chem., **177**, 993–994.

Kitay, E., and Snell, E. E. 1950 Some additional nutritional requirements of certain lactic acid bacteria. J. Bact. *In press.*

KLEIN, W. 1938 Über die Darstellung der Nucleoside aus Thymonucleinsäure. Z. physiol. Chem., **255**, 82–88.

KOCHER, V. 1949 Technique of the microbiological determination of vitamin B_{12} with *Lactobacillus lactis*. Intern. Z. Vitaminforsch., **20**, 369–374.

KOCHER, V., AND SCHINDLER, O. 1949 Desoxyribonucleoside as a growth factor for *Lactobacillus lactis* in B_{12}-free nutrient solution. Intern. Z. Vitaminforsch., **20**, 441–443.

KODITSCHEK, L. H., HENDLIN, D., AND WOODRUFF, H. B. 1949 Investigations on the nutrition of *Lactobacillus lactis* Dorner. J. Biol. Chem., **179**, 1093–1102.

LEVENE, P. A., AND BASS, L. W. 1931 Nucleic acids. Am. Chem. Soc. Monograph Series, New York.

MCNUTT, W. S., AND SNELL, E. E. 1948 Phosphates of pyridoxal and pyridoxamine as growth factors for lactic acid bacteria. J. Biol. Chem., **173**, 801–802.

MCNUTT, W. S., AND SNELL, E. E. 1950 Pyridoxal phosphate and pyridoxamine phosphate as growth factors for lactic acid bacteria. J. Biol. Chem., **182**, 557–567.

PIERCE, J. V., PAGE, A. C., JR., STOKSTAD, E. L. R., AND JUKES, T. H. 1949 Crystallization of vitamin B_{12b}. J. Am. Chem. Soc., **71**, 2952.

ROGOSA, M., TITTSLER, R. P., AND GEIB, D. S. 1947 Correlation of vitamin requirements and cultural and biochemical characteristics of the genus *Lactobacillus*. J. Bact., **54**, 13–14.

SAUBERLICH, H. E. 1949 The effect of folic acid upon the urinary excretion of the growth factor required by *Leuconostoc citrovorum*. J. Biol. Chem., **181**, 467–474.

SAUBERLICH, H. E., AND BAUMANN, C. A. 1948 A factor required for growth of *Leuconostoc citrovorum*. J. Biol. Chem., **176**, 165–173.

SAUBERLICH, H. E., AND BAUMANN, C. A. 1949 Further studies on the factor required by *Leuconostoc citrovorum* 8081. J. Biol. Chem., **181**, 871–877.

SHIVE, W., RAVEL, J. M., AND EAKIN, R. E. 1948 An interrelationship of thymidine and vitamin B_{12}. J. Am. Chem. Soc., **70**, 2614–2615.

SHORB, M. S. 1948 Activity of vitamin B_{12} for the growth of *Lactobacillus lactis*. Science, **107**, 397–398.

SKEGGS, H. R., HUFF, J. W., WRIGHT, L. D., AND BOSSHARDT, D. K. 1948 The use of *Lactobacillus leichmannii* in the microbiological assay of the "animal protein factor." J. Biol. Chem., **176**, 1459–1460.

SNELL, E. E., KITAY, E., AND MCNUTT, W. S. 1948 Thymine desoxyriboside as an essential growth factor for lactic acid bacteria. J. Biol. Chem., **175**, 473–474.

WELCH, A. D., AND WILSON, M. F. 1949 Mechanism of the growth-promoting effects of ascorbic acid on *Lactobacillus leichmannii* and the reduction of oxidation products of vitamin B_{12}. Arch. Biochem., **22**, 486–488.

WILLIAMS, W. L., BROQUIST, H. P., AND SNELL, E. E. 1947 Oleic acid and related compounds as growth factors for lactic acid bacteria. J. Biol. Chem., **170**, 619–630.

WILLIAMS, W. L., HOFF-JØRGENSEN, E., AND SNELL, E. E. 1949 Determination and properties of an unidentified growth factor for *Lactobacillus bulgaricus*. J. Biol. Chem., **177**, 933–940.