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Studies on the Folic Acid, Pyrimidine and Purine Requirements of Four Lactobacilli

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The specificity of the folic acid *, pyrimidine and purine requirements of four lactobacilli obtained from Moore and Rainbow [1] and isolated from the contaminant flora of English top fermentation brewer's yeast was investigated. The previous findings of these authors that the four strains designated L3, L4, L5, and L6 required pteroylmonoglutamic acid (PGA) **, a pyrimidine and purine(s) were confirmed. Further studies revealed that each of these requirements was non-specific and could be met using a range of related substances. The results obtained can be summarized as follows:

1. PGA could be replaced by N^5-formyltetrahydro-PGA, N^{10}-formyl-PGA, thymidine and TMP.
2. PABA was not essential in the presence of PGA or any compound replacing PGA.
3. The pyrimidine requirement of all four strains could be met using either uracil, uridine, 2′(3′)-UMP, deoxyuridine, cytosine, cytidine, 2′(3′)-CMP, deoxycytidine or deoxy-CMP.
4. The purine requirement of strain L4 could be met using either adenine, adenosine, 2′(3′)-AMP, deoxyadenosine, deoxy-AMP, hypoxanthine, inosine or deoxyinosine.
5. Strains L3, L5, and L6 could utilize as a sole purine source all the purine compounds active for strain L4 as well as guanine, guanosine, 2′(3′)-GMP, deoxyguanosine, deoxy-GMP, xanthine or xanthosine which were all inactive for strain L4.
6. Extracts of barley, kilned malt (without rootlets) and kilned malt rootlets, wort and beer were found to be active for replacing the folic acid, pyrimidine and purine requirements of all four lactobacilli.

Certain lactic acid bacteria have long been known to occur in association with brewery materials, *e.g.* malt, beer and yeast, but few studies have been carried out on their nutritional requirements. Recently, Russel, Bhandari and Walker

* "Folic acid" (factors) refers generally to the group of compounds chemically related to PGA which are capable of stimulating the growth of *Streptococcus faecalis* ATCC 8043 and *Lactobacillus casei* ATCC 7469; individual compounds will be specifically named.

** The following abbreviations are used in this paper: PGA for pteroylmonoglutamic acid; PABA for p-aminobenzoic acid; deoxy-CMP, TMP, deoxy-AMP, and deoxy-GMP for the 5′-monophosphates of deoxycytidine, thymidine, deoxyadenosine and deoxyguanosine; 2′(3′)-UMP, 2′(3′)-CMP, 2′(3′)-AMP and 2′(3′)-GMP for mixtures of the 2′- and 3′-monophosphates of uridine, cytidine, adenosine and guanosine, respectively.

studied the vitamin [2] and amino acid [3] requirements, and the interrelationships affecting the need for purines, PGA, and PABA [4] of 34 lactic acid bacteria isolated from the contaminant flora of English top fermentation beers and yeasts [5]. Moore and Rainbow [1] have studied the nutritional requirements of four strains of lactobacilli isolated from the contaminant flora of English top fermentation brewer's yeasts with respect to growth factors of the vitamin B-complex, purines, pyrimidines, amino acids and major sources of energy, and some biochemical activities. The strains were designated L3, L4, L5, and L6. Strains L3 and L4 were tentatively identified as strains of *Lactobacillus brevis*. According to Moore and Rainbow, all the strains required PGA, uracil and purines. [1] Through the courtesy of Dr. C. Rainbow, Department of Applied Biochemistry, University of Birmingham, we obtained cultures of the strains L3, L4, L5, and L6 for investigations on the replaceability of each of these growth requirements by a range of related substances. [6] In the present investigation, further studies of these and related problems have been made.

EXPERIMENTAL

Organisms. Since receiving the strains L3, L4, L5, and L6 from Dr. Rainbow in January 1956, stab cultures of these lactobacilli were maintained on Bacto micro assay culture agar (Difco) * and transferred at least once every two months. The cultures after incubation overnight at 30°C were stored in the refrigerator. Good growth also occurred in stab cultures on Bacto malt agar (Difco) but Bacto micro assay culture agar was superior.

Strain L6 was the slowest growing organism of the four brewery lactobacilli. The complete semisynthetic medium (see below) used in the tube tests did not give a good early growth with strain L6 and maximum growth did not occur until after 72 h of incubation at 28°C. Only 48 h or an even shorter time of incubation was required by strains L3, L4, and L5 in the tube tests. Sometimes broth subcultures of sufficient growth density were difficult to obtain with strain L6 and, in some tube test series, L6 unexpectedly failed to grow. These difficulties were also encountered to a certain degree with strain L4 whereas strains L3 and L5 were easy to work with.

Inocula. The inoculum medium consisted of equal volumes of single strength complete basal medium (see below) and Micro inoculum broth (Difco). Centrifuge tubes were filled with 5 ml portions of inoculum medium and a glass bead and were sterilized for 15 min at 120°C. Broth subcultures were incubated at 30°C for 18—24 h. For the preparation of inocula the subcultures were centrifuged, washed three times with 5 ml portions of sterile 0.9 % sodium chloride solution, resuspended and diluted in sterile sodium chloride solution to a turbidity of 65 % light transmittance read at 6 400 Å with a Coleman Model 11 Universal spectrophotometer. One ml of this suspension, corresponding to about 0.05 mg dry weight of cell material, was mixed with 50 ml sterile sodium chloride solution and, after thorough shaking, two drops of this dilute suspension was used to inoculate each tube.

Media. The complete medium used in these investigations was that of Moore and Rainbow [1] in a slightly modified form, having the following composition per 500 ml double strength medium: Bacto vitamin-free casamino acids (Difco), 10 g; L-cystine and L-cysteine hydrochloride, 100 mg each; DL-tryptophan, 200 mg; glucose and L-arabinose, 10 g each; sodium acetate (3 H_2O), 20 g; K_2HPO_4, $(NH_4)_2SO_4$, and NaCl, 1 g each; $MgSO_4 . 7H_2O$, 100 mg; $MnSO_4 . 4H_2O$, 30 mg; $FeSO_4 . 7H_2O$, 10 mg; adenine sulfate, guanine hydrochloride, and uracil, 10 mg each; thiamine hydrochloride, riboflavin, nicotinic acid, calcium pantothenate, and pyridoxine, 1 mg each; biotin, 10 μg; PABA, 500 μg; and PGA, 100 μg. The pH was adjusted to 6.5.

* Difco Laboratories, Inc., Detroit, Michigan, U.S.A.

As well as the complete medium, incomplete media lacking one or several essential factors were also used, *e. g.* purine deficient medium, (see below). All media were made up from stock solutions of each constituent as desired and the pH was adjusted to 6.5. The unsterilized double strength media were stored at −20°C. Media stored for 3−4 months have been successfully used.

Tube tests. Tube tests using a final volume of 10 ml were made in optically calibrated tubes (19 × 180 mm). A 5 ml amount of the factors or extracts to be tested was added to the test tubes, followed by 5 ml of double strength medium and a glass bead and the tubes were covered with aluminium caps. The tubes were sterilized by autoclaving at 120°C for 5 min. After cooling to room temperature in running tap water and inoculating (see above), the tubes were incubated at 28°C in a thermostated water bath. After 48, 72 and 96 h of incubation, growth was measured turbidimetrically as % light transmittance against uninoculated blanks set at 100 % light transmittance at 6 400 Å with a Coleman Model 11 Universal spectrophotometer. Since in most cases very little further growth occurred after 72 h of incubation, only the readings after 48 and 72 h of incubation are given in the tables. Each value in the tables is the mean of at least two separate tube tests. In each tube test two duplicate tubes were employed. The readings of identical tube tests made on separate occasions usually agreed very well. When there was poor agreement the tube test was repeated until consistent results were obtained.

Biochemicals. PGA (Folvite), N^5-formyltetrahydro-PGA (synthetic folinic acid, leucovorin), pteroic acid and N^{10}-formylpteroic acid (rhizopterin) were gifts from Lederle Laboratories; N^{10}-formyl-PGA (rhizopterin glutamate) was a gift from Hoffman-La Roche. The pyrimidine and purine derivatives were obtained from the California Corporation for Biochemical Research, Los Angeles, California. The deoxyribonucleotides are the 5′-phosphate esters while the ribonucleotides are mixtures of the 2′- and 3′-phosphate esters. Adenine and cytidine were supplied as hemisulfates, guanine and deoxycytidine as hydrochlorides, deoxy-AMP and deoxy-GMP as the monoammonium salts, TMP as dihydrate of the calciumsalt, 2′(3′)-GMP as dihydrate of the trisodium salt, 2′(3′)-AMP and 2′(3′)-UMP as the dihydrates, and uridine as the monohydrate; all other nucleic acid derivatives were supplied as pure compounds without water of crystallisation etc.

Purity of deoxyuridine, deoxycytidine, thymidine, and TMP. The purity of these deoxyribose compounds was checked by bioautography of paper chromatograms. Two solvent systems were used: (I) *sec.* butanol-water-conc. ammonia (80:20:3 v/v) and (II) *sec.* butanol-water-conc. acetic acid (75:25:3 v/v). The following R_F values were found in solvent system (I): folinic acid factors * and deoxyribonucleotides 0, deoxycytidine and deoxyuridine 0.35, thymidine 0.56; and in solvent system (II): deoxyribonucleotides 0.04−0.10, deoxycytidine 0.38, deoxyuridine 0.53, thymidine 0.65 and folinic acid factors 0.20−0.55. For bioautography, agar plates inoculated with either *Pediococcus cerevisiae (Leuconostoc citrovorum)* ATCC 8081 or *Lactobacillus leichmannii* 313 ATCC 7830 were used. The plates were made according to the procedure reported earlier for the plate assay of folinic acid factors with *P. cerevisiae* [7]. For both test organisms, the *P. cerevisiae* agar medium was used but the amounts of glucose and sodium acetate were doubled. After incubation overnight at 37°C, the plates were read. *P. cerevisiae* responds to thymidine and folinic acid factors whereas *Lb. leichmannii* responds to vitamin B_{12} (and factors), deoxyribonucleosides and deoxyribonucleotides.

Bioautography of moderate amounts (about 10 μg) of deoxyuridine revealed that it was contaminated by a small amount of thymidine. Quantitative work (quantitative paper chromatography combined with microbiological tube assays with *P. cerevisiae* and/or *Lb. leichmannii*) showed that deoxyuridine was contaminated by 0.15 % of thymidine (the mean value obtained from three separate determinations). On the other hand, thymidine was found to be contaminated by 1.5 % of deoxyuridine (only one determination was made). Furthermore, bioautography of large amounts (about 40 μg) of deoxycytidine and TMP revealed that these compounds were contaminated by faint traces (less than 0.02 %) of deoxyuridine and traces (about 0.05 %) of thymidine, respectively. According to the suppliers, these four deoxyribose compounds were guaranteed to have a purity of 100 ± 2 % as tested spectrophotometrically.

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* "Folinic acid factors" designates those folic acid factors that are closely related to N^5-formyltetrahydro-PGA and are capable of stimulating the growth of *P. cerevisiae*.

Extracts of brewery products. The same brewery products were used as previously investigated [6]. Water extracts of kilned malt (without rootlets) and kilned malt rootlets were made by mixing 5 g of the finely ground (Wiley mill) material with 40 ml 0.1 M phosphate buffer at pH 6.5. After heating at 100°C for 10 min, the volumes were made up to 100 ml with distilled water and centrifuged. Water extracts of barley were made by mixing 5 g of barley with 40 ml 0.1 M phosphate buffer at pH 5.8. The mixture was first homogenized (MSE-homogenizer) and then heated at 100°C for 10 min. After cooling, the starchy mixture was liquefied by treatment for 4 h at 37°C with a bacterial amylase preparation (50 mg of Rapidase). The enzyme action was terminated by steaming for 10 min. The pH was then adjusted to 6.5 and the volume made up to 100 ml with distilled water and centrifuged. Samples of wort (12.5 % extract) and beer (made from this wort) from a top yeast fermentation were treated as follows: to 80 ml of sample were added 5 ml 1 M phosphate buffer, the pH adjusted to 6.5 and the volume made up to 100 ml. After heating at 100°C for 10 min and then cooling, the mixtures were filtered if necessary.

Table 1. Response of lactobacilli strains L3, L4, L5, and L6 in a PGA deficient medium to some folic acid factors and pyrimidine derivatives and extracts of brewery materials [a].

Addition to deficient medium	Amount per tube of 10 ml	L3 Incubation 48 h	L3 Incubation 72 h	L4 Incubation 48 h	L4 Incubation 72 h	L5 Incubation 48 h	L5 Incubation 72 h	L6 Incubation 48 h	L6 Incubation 72 h
None		100	100	100	100	100	100	100	100
PGA	0.001 μg	30	7	100	90	70	70	100	100
	0.01 μg	6	4	80	8	8	8	100	97
	1 μg[b]	3	2	5	3	5	4	15	6
N^5-Formyltetra-hydro-PGA	0.01 μg	8	4	100	30	10	9	100	32
	1 μg	5	3	20	4	6	6	50	5
N^{10}-Formyl-PGA	0.01 μg	5	4	70	7	11	11	100	70
	1 μg	4	3	15	4	7	5	80	13
Thymidine	1 μg	44	25	75	58	46	51	83	65
	10 μg	29	5	42	6	20	12	83	22
	100 μg	22	4	23	4	13	4	73	19
TMP	100 μg	43	10	40	9	60	22	80	30
Extract of barley	50 mg[c]	4	3	77	76	54	54	50	5
Extract of kilned malt (without rootlets)	50 mg[c]	3	3	35	14	6	5	85	30
Extract of kilned malt rootlets	10 mg[c]	3	3	9	8	4	4	65	45
Wort	0.1 ml[d]	4	3	9	8	5	5	27	26
Beer	0.1 ml[d]	3	2	4	3	4	4	24	26

[a] Turbidity readings: % light transmittance; no growth = 100 % transmittance; maximum growth = 2 % transmittance.
[b] Represents composition of complete medium.
[c] Dry weight of original sample.
[d] Original sample.

RESULTS AND DISCUSSION

Folic acid requirement. Moore and Rainbow [1] showed that all four strains required PGA but did not investigate the specificity of this requirement. We found that the PGA requirement of all four strains was quite nonspecific and could be met by any one of N^5-formyltetrahydro-PGA, N^{10}-formyl-PGA, thymidine, TMP or extracts of brewery materials. The growth promoting effects of these compounds and extracts after 48 and 72 h of incubation in a PGA deficient medium are given in Table 1. Omission of PABA from the PGA deficient medium gave the same growth for all compounds and extracts tested in Table 1. PABA was therefore not essential in the presence of PGA or any of the compounds and extracts that were able to replace PGA in the nutrition of these four lactobacilli.

Of the folic acid factors tested, PGA, N^5-formyltetrahydro-PGA, and N^{10}-formyl-PGA were about equally active for all four strains whereas pteroic acid and N^{10}-formylpteroic acid were inactive (see below). Strains L3 and L5 had the smallest folic acid requirement and the complete medium contained over a 100-fold excess of the amount of PGA required for optimum growth for these two strains. Strain L4 and particularly strain L6 had a much higher folic acid requirement than strains L3 and L5. Thymidine and TMP, which also promoted growth for all four strains in a PGA deficient medium, were required in much larger amounts than PGA for obtaining similar growth levels. These two compounds were about equally active on a molar basis.

The following compounds were unable to promote growth in a PGA deficient medium (containing PABA) for all four lactobacilli after 96 h of incubation when tested in the amount per tube given in brackets: pteroic acid (10 μg), N^{10}-formylpteroic acid (1 and 10 μg), thymine (100 and 1 000 μg), uridine (100 and 1 000 μg), 2′(3′)-UMP (100 and 1 000 μg), cytosine (100 and 1 000 μg), cytidine (100 μg), 2′(3′)-CMP (100 μg), deoxycytidine (100 and 500 μg), deoxy-CMP (100 μg), orotic acid (500 μg), deoxyadenosine (100 μg), deoxyguanosine (100 μg), Tween 80 (5 mg) and vitamin B_{12} (0.1 μg).

Somewhat irregular results were obtained when testing the growth promoting effect of deoxyuridine (not tabulated). We reported earlier that 100 μg of deoxyuridine per tube was inactive for all four lactobacilli in PGA deficient medium [6]. Repeated tube tests showed that strain L5 occasionally responded to 100 μg of deoxyuridine and that all four strains responded to 500 μg of deoxyuridine. However, the turbidity readings of repeated experiments were not sufficiently uniform to merit inclusion in Table 1. In all these cases, the growth promoting effect of deoxyuridine was too high to account for the low trace contamination by 0.15 % thymidine. A possible explanation of the irregular growth promoting effect of large amounts of deoxyuridine could be that an uneven carry-over of PGA occurred when inoculating the tubes and that this resulted in a varying degree of formation of thymidine (or TMP) from deoxyuridine. Separate experiments showed that resting cells of strain L5 were able to convert about 1 % of added deoxyuridine to thymidine (mean value of four resting cell experiments) when incubated in buffered glucose-arabinose solution at 28°C for 4 h.

Growth experiments were also made with strain L5 in an attempt to demonstrate the presence of trans-N-deoxyribosylase [8,9] but these tests were negative. No growth of strain L5 after 72 h of incubation was found in a PGA deficient medium with thymine (1 000 μg) + deoxyuridine (100 μg) or thymine (1 000 μg) + deoxycytidine (100 μg).

All the extracts of brewery materials tested were found to be active in replacing the folic requirement for all four lactobacilli. This finding was expected since earlier investigations from this laboratory had shown that these brewery materials contained folic acid factors and thymidine [6,10]. In particular, kilned malt rootlets were found to be rich in both folic acid factors and thymidine. As seen from Table 1, the extract of kilned malt rootlets was more active on a dry weight basis than the extract of kilned malt (without rootlets) and the extract of barley for strains L3, L4, and L5. The reverse order of activity of these extracts was, however, found for strain L6. Wort and beer were very active and both had almost the same growth promoting effect in a PGA deficient medium for all four lactobacilli.

The specificity of the folic acid requirement of lactobacilli varies from organism to organism. The folic acid requirement of these four brewery lactobacilli resembled that of *Lactobacillus casei* ATCC 7469 in many respects [11,12]. Thus, *Lb. casei* as well as the four brewery lactobacilli require the complete folic acid molecule with the glutamic acid group for growth; pteroic acid and N^{10}-formylpteroic acid are completely or almost completely inactive. Thymine is inactive for the four brewery lactobacilli but is active in replacing PGA for *Lb. casei* [11,13]. Thymidine, on the other hand, promotes growth in a PGA deficient medium for all five of these organisms [11,13]. However, none of the thirty-four brewery lactobacilli investigated by Russell *et al.* [2] had any requirement for PGA but one organism required PABA. The method used by Russel *et al.* [2] for determining the vitamin requirements of lactobacilli, which depended upon the evidence of a single transfer into the deficient media, has been critisized by Ford *et al.* [14] who recommend at least two or three transfers when assessing the vitamin requirements of bacteria, particularly when requirements for vitamin B_6, folic acid and biotin are being studied.

Pyrimidine requirement. Moore and Rainbow [1] showed that the pyrimidine requirement of all four lactobacilli could be met by uracil or cytosine but not by thymine. Strains L3, L5, and L6 could utilize orotic acid for growth in a pyrimidine deficient medium. These findings were confirmed. We further found that the pyrimidine requirement of all four strains could be met by any one of uridine, 2′(3′)-UMP, deoxyuridine, cytidine, 2′(3′)-CMP, deoxycytidine, deoxy-CMP or extracts of brewery materials. The growth promoting effects of these compounds and extracts after 48 and 72 h of incubation are given in Table 2. Uracil and cytosine were the most active of these compounds, whereas the ribonucleotides had the lowest activity. Orotic acid had the same growth promoting activity as uracil or cytosine for strains L3, L5, and L6 whereas strain L4 could not utilize orotic acid.

The following compounds were unable to promote growth in a pyrimidine deficient medium for all four lactobacilli after 96 h of incubation when tested in the amounts per tube given in brackets: N^5-formyltetrahydro-PGA (10 μg), N^{10}-formyl-PGA (10 μg) thymine (100 and 1 000 μg), thymidine (100 μg),

Table 2. Response of lactobacilli strains L3, L4, L5, and L6 in a pyrimidine deficient medium to pyrimidine derivatives and extracts of brewery materials.[a]

Addition to deficient medium	Amount per tube of 10 ml	L3 Incubation 48 h	L3 Incubation 72 h	L4 Incubation 48 h	L4 Incubation 72 h	L5 Incubation 48 h	L5 Incubation 72 h	L6 Incubation 48 h	L6 Incubation 72 h
None		100	100	100	100	100	100	100	100
Uracil	10 μg[b]	68	64	80	62	54	44	64	59
	100 μg	3	2	5	3	5	4	15	6
Uridine	100 μg	15	10	88	9	11	10	66	12
2′(3′)-UMP	100 μg	28	20	70	33	19	14	80	27
Deoxyuridine	100 μg	8	5	13	9	9	7	20	7
Orotic acid	100 μg	4	3	100	100	4	3	60	7
Cytosine	100 μg	3	2	68	11	5	4	14	4
Cytidine	100 μg	23	16	80	23	10	8	23	13
2′(3′)-CMP	100 μg	40	33	61	33	26	16	39	28
Deoxycytidine	100 μg	16	10	25	17	14	9	23	11
Deoxy-CMP	100 μg	12	8	17	11	13	9	19	12
Extract of barley	50 mg[c]	85	85	87	84	82	82	82	78
Extract of kilned malt (without rootlets)	50 mg[c]	71	66	68	53	46	36	77	58
Extract of kilned malt rootlets	10 mg[c]	67	63	41	38	33	25	60	38
Wort	0.1 ml[d]	64	60	65	58	57	48	65	64
Beer	0.1 ml[d]	63	59	67	63	54	48	60	60

[a] Turbidity readings: % light transmittance; no growth = 100 % transmittance; maximum growth 2 % transmittance.
[b] Represents composition of complete medium.
[c] Dry weight of original sample.
[d] Original sample.

TMP (100 and 500 μg), Tween 80 (5 mg), and vitamin B_{12} (0.1 μg). When testing 1 000 μg of thymidine per tube, a growth response was obtained for all four strains. However, the contamination of thymidine by 1.5 % of deoxyuridine could completely account for this growth promoting effect of thymidine in the pyrimidine deficient medium.

All the extracts of brewery materials tested were found to be active in replacing the pyrimidine requirement for the four lactobacilli. Extract of kilned malt rootlets was found to be more active on dry weight basis than either of kilned malt (without rootlets) or barley for all four strains. Wort and beer each had the same activity for all four strains. We have shown earlier [6,10] that kilned malt rootlets, wort and beer contained a growth factor for *Lb. leichmannii* 313 ATCC 7830 and *Lb. acidophilus* R-26 ATCC 11506 with the same R_F-value as deoxyuridine in several chromatographic solvent systems.

Russell *et al.* [4] investigated the uracil requirement of thirty-four brewery lactobacilli and found that uracil was essential for seventeen organisms and

stimulatory to four others. The specificity of the uracil requirement of these brewery lactobacilli was not investigated. For pyrimidine requiring lactobacilli the specificity of the requirement for pyrimidine varies greatly from organism to organism. In several instances, it has been shown that lactobacilli can meet their pyrimidine requirement by means of several closely related pyrimidines or pyrimidine derivatives. [15-18]

Purine requirement. Moore and Rainbow [1] showed that the purine requirement of strains L3, L5, and L6 could be met by adenine, guanine, xanthine or hypoxanthine. Strain L4 required hypoxanthine alone or adenine together with either guanine or xanthine [1]. However, the purine requirement of strain L4 has changed somewhat with time. This strain will now also grow on adenine alone (private communication from Dr. Rainbow). We confirmed these findings. Further investigations revealed that the purine requirement of all four lactobacilli could also be met by any one of several purine derivatives or extracts of brewery materials. The growth promoting effects of these compounds and extracts after 48 and 72 h of incubation are given in Table 3.

As seen from Table 3, strains L3, L5, and L6 could utilize any one of the purine derivatives tested as a sole purine source. Strain L4, on the other hand, could only utilize adenine, adenosine, 2′(3′)-AMP, deoxyadenosine, deoxy-AMP, hypoxanthine, inosine or deoxyinosine as a sole purine source. Extracts of brewery materials were active for all four strains.

For all four strains, the highest growth levels (2—10 % light transmittance) were obtained with adenine + guanine (100 μg of each) or deoxyadenosine + deoxyguanosine (100 μg of each) after 72 h of incubation. Strains L3 and L5 were similar in the utilization of individual purine compounds. For both strains, the highest growth levels were also obtained with 100 μg of any one of the purines or adenosine whereas the remaining purine nucleosides had the highest growth levels for strain L3 and good growth (10—20 % light transmittance) for strain L5. The purine nucleotides were less active on a molar basis for both strains.

For strain L4, no single purine compound could duplicate the high growth promoting effect of adenine + guanine or deoxyadenosine + deoxyguanosine. The compounds most active in promoting the growth of strain L4 in a purine deficient medium were adenosine, hypoxanthine, inosine and deoxyinosine followed by deoxyadenosine and deoxy-AMP. Considerably less active for strain L4 on a molar basis were adenine and 2′(3′)-AMP whereas guanine, xanthine and their derivatives were completely inactive. Although guanine alone was inactive, it greatly enhanced the growth promoting effect of adenine. Deoxyguanosine, which was also inactive alone, enhanced the growth promoting effect of deoxyadenosine.

For strain L6, the highest growth levels (after 72 h of incubation) were also obtained with hypoxanthine, inosine or deoxyinosine. Of the remaining purine bases, only guanine gave good growth whereas adenine and xanthine had low growth promoting effects. However, adenine had a synergistic growth effect together with guanine. For all four strains, purine nucleosides were more active growth promotors than purine nucleotides.

The following compounds were unable to promote growth in a purine deficient medium for all four strains after 96 h of incubation when tested in the

Table 3. Response of lactobacilli strains L3, L4, L5, and L6 in a purine deficient medium to purine derivatives and extracts of brewery materials [a].

Addition to deficient medium	Amount per tube of 10 ml	L3 Incubation 48 h	L3 Incubation 72 h	L4 Incubation 48 h	L4 Incubation 72 h	L5 Incubation 48 h	L5 Incubation 72 h	L6 Incubation 48 h	L6 Incubation 72 h
None		100	100	100	100	100	100	100	100
Adenine	10 μg	67	58	96	94	87	71	98	95
	100 μg	6	4	85	40	7	7	85	70
Adenosine	100 μg	9	5	65	7	7	6	90	11
2′(3′)-AMP	100 μg	24	14	82	36	38	20	89	50
Deoxyadenosine	100 μg	17	8	22	14	16	16	39	16
Deoxy-AMP	100 μg	54	36	43	16	22	22	44	22
Guanine	10 μg	60	56	100	100	76	74	90	70
	100 μg	6	4	100	100	11	10	27	13
Guanosine	100 μg	9	7	100	100	19	19	90	24
2′(3′)-GMP	100 μg	25	14	100	100	40	36	90	44
Deoxyguanosine	100 μg	10	7	100	100	17	17	40	15
Deoxy-GMP	100 μg	14	9	100	100	24	24	44	18
Adenine + Guanine	100 μg + 100 μg [b]	3	2	5	3	5	4	15	6
Deoxyadenosine + + Deoxyguanosine	100 μg + 100 μg	5	4	14	5	5	5	75	10
Hypoxanthine	100 μg	5	3	30	9	7	7	92	9
Inosine	100 μg	7	5	35	11	15	15	15	5
Deoxyinosine	100 μg	8	5	26	12	13	13	17	7
Xanthine	100 μg	5	3	100	100	7	6	96	65
Xanthosine	100 μg	9	9	100	100	20	20	88	28
Extract of barley	50 mg [c]	74	74	78	65	75	73	81	69
Extract of kilned malt (without rootlets)	50 mg [c]	46	35	60	33	41	32	94	42
Extract of kilned malt rootlets	10 mg [c]	30	23	32	23	30	24	60	32
Wort	0.1 ml [d]	47	42	82	76	65	59	70	58
Beer	0.1 ml [d]	49	45	84	80	65	60	60	58

[a] Turbidity readings: % light transmittance; no growth = 100 % transmittance; maximum growth 2 % transmittance.
[b] Represents composition of complete medium.
[c] Dry weight of original sample.
[d] Original sample.

amounts per tube given in brackets: thymidine (1 000 μg), deoxyuridine (500 μg) and vitamin B_{12} (0.1 μg).

All extracts of brewery materials tested were found to be active in replacing the purine requirement for all four strains. Extract of kilned malt rootlets was

found to be more active on dry weight basis than either of kilned malt (without rootlets) or barley, just as was the case when these extracts replaced the pyrimidine requirement for all four strains. Wort and beer each had the same activity. Extracts of brewery materials are known to contain purines and purine derivatives. We have shown earlier [6,10] that kilned malt rootlets, wort, and beer contain an acid labile growth factor for *Lb. leichmannii* 313 and *Lb. acidophilus* R-26 with the same R_F-value as deoxyguanosine in several solvent systems. Harris and Parsons [19,20] investigated the purine derivatives present in wort and found that the greater part of these compounds consists of adenosine and guanosine. Deoxyadenosine and deoxyguanosine as well as the free purines adenine and guanine were also identified.

The specificity of the purine requirement of lactobacilli varies greatly from organism to organism and is intimately related to their pyrimidine, folic acid, and vitamin B_{12} requirements [16,21,22]. It should be pointed out that the four strains of brewery lactobacilli investigated here had absolute requirements for folic acid, a pyrimidine and a purine. In no instance, could these requirements be met using vitamin B_{12}.

Russell *et al.*[4] investigated the purine requirements of several brewery lactobacilli by omitting in turn each of the purines, adenine, guanine and xanthine, from the complete medium which contained PGA, PABA, and uracil. Of the thirty-four organisms investigated, twenty-six were unaffected by the omissions of any one of the purines. Adenine was found to be essential to seven organisms. Guanine was essential to one organism and stimulatory to two whereas xanthine was not required by any strain and stimulated the growth of only one strain. The interrelationships in the requirements for purines, PGA, PABA, and uracil were investigated for some of the strains. However, the specificity of the purine requirement of these lactobacilli, found in the presence of PGA, PABA, and uracil, was not investigated.

Combined folic acid, pyrimidine, and purine requirements. The four strains of lactobacilli under investigation had absolute requirements for folic acid, a pyrimidine and a purine. As shown above, each of these requirements could be met by an appropriate deoxyribonucleoside. It was therefore considered of interest to measure the growth promoting effects of various combinations of deoxyribonucleosides in a medium deficient in PGA, PABA, pyrimidines and purines. Extracts of brewery materials were also tested. The turbidity readings after 48 and 72 h of incubation are given in Table 4.

The combination of the four deoxyribonucleosides, deoxyadenosine + deoxyguanosine + deoxyuridine + thymidine (100 μg of each), gave nearly optimum growth for all four strains. Only certain combinations of two or three deoxyribonucleosides gave good growth for some of the strains: for strain L5, the combination of deoxyguanosine + deoxyuridine (100 μg + 500 μg) and, for strains L3 and L5, the two combinations of deoxyadenosine + deoxyuridine + thymidine (100 μg of each) and deoxyguanosine + deoxyuridine + thymidine (100 μg of each). The response of strain L4 to the various combinations of deoxyribonucleosides was in accordance with the previous finding that deoxyguanosine alone could not meet the purine requirement of this strain. As seen from Table 4, the combination of deoxyadenosine + deoxyguanosine + deoxyuridine (100 μg of each) was inactive or almost

Table 4. Response of lactobacilli strains L3, L4, L5, and L6 in a medium deficient in PGA, PABA, pyrimidines and purines to combinations of deoxyribonucleosides and to extracts of brewery materials [a].

Addition to deficient medium	Amount per tube of 10 ml	L3 Incubation 48 h	L3 Incubation 72 h	L4 Incubation 48 h	L4 Incubation 72 h	L5 Incubation 48 h	L5 Incubation 72 h	L6 Incubation 48 h	L6 Incubation 72 h
Deoxyadenosine + + thymidine	100 μg + 500 μg	86	76	100	96	77	62	91	76
Deoxyguanosine + + thymidine	100 μg + 500 μg	75	64	100	100	78	70	95	85
Deoxyguanosine + + deoxyuridine	100 μg + 500 μg	92	80	100	100	36	18	97	96
Deoxyadenosine + + deoxyuridine + + thymidine	100 μg each	62	28	97	75	24	16	90	44
Deoxyguanosine + + deoxyuridine + + thymidine	100 μg each	52	16	100	100	35	20	85	41
Deoxyadenosine + +deoxyguanosine +deoxyuridine	100 μg each	95	95	100	100	98	90	100	100
Deoxyadenosine + +deoxyguanosine + +deoxyuridine + +thymidine	100 μg each	23	5	27	6	10	5	78	18
Extract of barley	50 mg[b]	82	82	91	80	78	75	90	80
	100 mg	81	81	80	74	70	62	90	80
Extract of kilned malt (without rootlets)	50 mg[b]	67	68	69	50	54	31	72	43
	100 mg	62	61	43	20	25	16	57	25
Extract of kilned malt rootlets	10 mg[b]	65	65	65	30	24	19	47	26
	50 mg	8	5	10	5	4	4	5	5
Wort	0.1 ml[c]	52	50	82	70	76	65	69	59
	1.2 ml	3	2	8	4	3	3	5	3
Beer	0.1 ml[c]	55	53	86	56	76	71	68	60
	1.2 ml	3	3	14	6	4	4	6	4

[a] Turbidity readings: % light transmittance; no growth = 100 % transmittance; maximum growth ? % transmittance.
[b] Dry weight of original sample.
[c] Original sample.

inactive for all four strains. This showed that PGA or a compound active in replacing PGA was still required when the purine and pyrimidine requirements were met using suitable deoxyribonucleosides.

Extract of kilned malt rootlets (50 mg), wort (1.2 ml), and beer (1.2 ml) gave optimum growth for all four strains. For strain L6, the turbidity readings after 48 h of incubation with these extracts exceeded those obtained with the complete semisynthetic medium. This difference was more pronounced when the turbidities were compared at shorter incubation times than 48 h. This showed that these extracts contained factors that promoted the early growth of strain L6. For all four strains, extract of barley (100 mg) gave poor growth whereas extract of kilned malt (100 mg) gave good or quite good growth. This indicates that to a certain extent there was a formation of important growth factors for these four lactobacilli during the malting process. A comparison of the growth promoting effects of extract of kilned malt (50 and 100 mg) and of wort (0.1 and 1.2 ml) reveals that, during the mashing process, there was a much greater formation of these growth factors than during the malting process [6] (0.1 and 1.2 ml of wort correspond to about 17 and 200 mg of malt, respectively, since in the brewing process about 6 ml of wort is obtained per gram of malt).

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