Metabolism of 5-Methyltetrahydrofolate by *Lactobacillus casei*

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The metabolism of 5-[Me-¹⁴C]methyltetrahydrofolate in *Lactobacillus casei* proceeded oxidatively with incorporation of label into purine and thymidylate derivatives. No labelled methionine was formed. (l)-5-Methyltetrahydrofolate, the natural isomer, was not a substrate for the *L. casei* folylpoly- γ -glutamate synthetase although the unnatural (d)-isomer was slowly metabolized to the di glutamate form.

INTRODUCTION

We have previously shown that pteroylpolyglutamates, once transported, are better growth promoters of *Lactobacillus casei* than pteroylmonoglutamates (Shane & Stokstad, 1976). The rate-limiting step in the utilization of pteroylpolyglutamates by this organism is transport into the cell, while intracellular metabolism, and not transport, is rate-limiting with pteroylmonoglutamates. *In vitro* studies have also shown that pteroylpolyglutamates are often better substrates than the corresponding monoglutamates for folate-requiring enzymes (Burton & Metzenberg, 1975; Cheng, Shane & Stokstad, 1975; Coward *et al.*, 1974, 1975; Curthoys & Rabinowitz, 1972; Kisliuk, Gaumont & Baugh, 1974; Powers & Snell, 1976; Salem, Pattison & Foster, 1972; Whitfield, Steers & Weissbach, 1970).

To investigate which step in pteroylmonoglutamate metabolism might be rate-limiting, the metabolism of 5-[Me-¹⁴C]methyl-H₄PteGlu and 5-methyl-H₄[³H]PteGlu (for nomenclature, see Methods) were compared in order to assess the turnover of intracellular folate via various metabolic pathways. Although two mechanisms have been described for 5-methyl-H₄PteGlu formation in bacteria involving the reduction of 5,10-methylene-H₄PteGlu or the direct transfer of a methyl group from trimethylsulphonium to H₄PteGlu (Wagner *et al.*, 1967), the only known route for the metabolism of methyltetrahydrofolates is via methionine synthesis (Taylor & Weissbach, 1973) but attempts to detect methionine synthetase activity in *L. casei* have proved unsuccessful (Galivan, 1971; Kisliuk, 1971).

METHODS

Nomenclature. The abbreviations used are: PteGlu, pteroylglutamic acid, folic acid; PteGlu_n, pteroylmono- to pteroyl oligo- γ -L-glutamic acid, *n* indicating the number of glutamic acid residues; H₄PteGlu_n, 5,6,7,8-tetrahydropteroylmono- to 5,6,7,8-tetrahydropteroyl oligo- γ -L-glutamic acid. The symbols (l) and (d) are used to denote the natural and unnatural diastereoisomers of H₄PteGlu_n, respectively, due to the asymmetric centre at the C-6 position, and do not indicate optical activity.

Materials. (l)-5-[Me-¹⁴C]Methyl-H₄PteGlu_n (*n* = 1, 3, 5 and 7) (specific activity 47 mCi mmol⁻¹), (dl)-5-methyl-H₄[³H]PteGlu (sp. act., 500 mCi mmol⁻¹), and folic acid γ -polyglutamates PteGlu₂₋₇, [³H]PteGlu₂₋₇ (sp. act., 4 mCi mmol⁻¹), and PteGlu_n-[¹⁴C]Glu-Glu (*n* = 0, 1, 2 and 3) (sp. act., 100 to 500 μ Ci mmol⁻¹) were synthesized and purified as described previously (Shane & Stokstad, 1976). (dl)-5-[Me-¹⁴C]Methyl-H₄PteGlu (sp. act., 54 mCi mmol⁻¹), L-[Me-¹⁴C]methionine (sp. act., 56 mCi mmol⁻¹) and S-adenosyl-L-[Me-¹⁴C]methionine (sp. act., 55 mCi mmol⁻¹) were obtained from Amersham/Searle (Arlington Heights, Illinois, U.S.A.); [8-³H]adenine (sp. act., 14 Ci mmol⁻¹), [8-³H]guanine (sp. act., 5.5 Ci mmol⁻¹), [6-³H]uracil

(sp. act., 20 Ci mmol⁻¹), [5-³H]cytosine (sp. act., 26 Ci mmol⁻¹) and [6-³H]thymine (sp. act., 7·1 Ci mmol⁻¹) were from Schwarz/Mann (Orangeburg, New Jersey, U.S.A.).

Unlabelled purine and pyrimidine derivatives were obtained from Sigma, DNAase (bovine pancreas; 1500 units mg⁻¹) from Worthington, and RNAase (bovine pancreas; 64 units mg⁻¹) and RNAase-CB (predominantly T₂, remainder T₁; 21 units mg⁻¹) from Calbiochem.

Organism and growth conditions. *Lactobacillus casei* (ATCC7469) was cultured as described previously (Tamura *et al.*, 1972) with PteGlu (2·3 mM) added to the media.

Uptake and metabolism of folates, purine and pyrimidine bases, and methionine. Bacteria were harvested by centrifuging from growth media in late exponential phase (20 to 24 h at 37 °C) and were washed with, and resuspended in 50 mM-K₂HPO₄/100 mM-sodium acetate, adjusted to pH 6 with H₃PO₄, and containing 1 % (w/v) glucose and 5 mM-mercaptoethanol. Transport of labelled bases and methionine was measured as described previously for folates (Shane & Stokstad, 1975, 1976). Bacterial dry weight was estimated by E_{640}^{opt} .

For metabolism studies, *L. casei* suspensions (0·2 mg dry wt ml⁻¹) in acetate/phosphate buffer pH 6 (containing glucose and mercaptoethanol), were preincubated for 15 min at 37 °C with labelled methyl-tetrahydrofolates or methionine, then filtered (HA filters, Millipore Corp., 0·45 µm pore size), washed with buffer, resuspended in fresh buffer (0·4 mg dry wt ml⁻¹), and incubated at 37 °C with shaking. Samples (5 ml) were removed at 0, 1, 2, 5 and 24 h and filtered, and the bacteria (plus filter) were resuspended in 0·1 M-potassium phosphate buffer pH 7, containing 0·2 M-mercaptoethanol. Intracellular labelled compounds were extracted by boiling for 5 min, and insoluble material was removed by centrifuging and resuspended in water (5 ml). Mercaptoethanol was added to the cell-free medium.

The metabolism of labelled purine and pyrimidine bases was studied in a similar manner except that the bacteria were preincubated with the ³H-labelled bases (5 µCi ml⁻¹) for 2 h before incubating with (*D*)-5-[Me-¹⁴C]methyl-H₄PteGlu (0·5 µM) for 15 min. After resuspension and incubation in fresh buffer for 2 h, the bacteria were filtered, washed, and extracted with boiling 0·1 M-potassium phosphate buffer pH 7, containing 0·2 M-mercaptoethanol (2 ml). Insoluble material, collected by centrifuging, was washed 10 times with H₂O (5 ml), resuspended in 20 mM-acetate buffer pH 4·5 (1 ml), containing RNAase (50 units) and RNAase T₂ (15 units), and incubated at 37 °C for 12 h. The mixtures were heated at 100 °C for 5 min, cooled, centrifuged and the supernatants (termed the RNAase extracts) were collected. Extracellular extracts, after addition of mercaptoethanol (0·2 M), were adjusted to pH 4 with acetic acid. Active charcoal (50 mg ml⁻¹) was added and the mixtures were stirred for 15 min. The charcoal was then collected by centrifuging, washed twice with H₂O, and extracted with 60 % (v/v) ethanol containing 0·1 M-NH₄OH (50 ml g⁻¹). Charcoal was removed by filtration and the ethanol extract was evaporated to dryness under reduced pressure at 40 °C. The residue was dissolved in 0·1 M-potassium phosphate buffer pH 7, containing 0·2 M mercaptoethanol (2 ml).

Portions of the various fractions were added to vials together with a Triton X-100/toluene (1:2, v/v) scintillation mixture (Goldmark & Linn, 1970). Counting efficiencies, determined by external standardization or by a channels ratio method, were about 80 % for ¹⁴C and 25 % for ³H. In dual labelling experiments, the ¹⁴C efficiency was about 35 %.

Identification of labelled compounds. Extracts were chromatographed on Sephadex G-25 and DEAE-cellulose (DE52, Whatman) before and after folyl-γ-glutamyl carboxypeptidase (conjugase) treatment as previously described (Buehring, Tamura & Stokstad, 1974; Shane & Stokstad, 1975; Tamura *et al.*, 1972). Individual labelled peaks were rechromatographed together with appropriate standards. The chromatographic behaviour of folates on Sephadex G-25 and DEAE-cellulose has been described in detail (Buehring *et al.*, 1974; Shin, Buehring & Stokstad, 1972a; Shin, Williams & Stokstad, 1972b).

Identification of 3'-ribonucleotides. 3'-Ribonucleotide standards were added to the RNAase extracts and portions were lyophilized to dryness, resuspended in H₂O (20 µl) and applied to cellulose thin-layer plates (Eastman 13255). Ribonucleotides were separated by two-dimensional chromatography using isobutyric acid/0·5 M-NH₄OH (5:3, v/v) and 2-propanol/6 M-HCl (7:3, v/v) as solvent systems (Delk & Rabinowitz, 1975; Nishimura, 1972). Standard compounds were detected by ultraviolet absorption (254 nm) and labelled compounds by scintillation autoradiography (Contractor & Shane, 1969; Klagsbrun, 1973).

Treatment of kinetic data. K_m and V_{max} values were calculated by an unweighted non-linear regression method (Wilkinson, 1961) with six cycles of reiteration (Cleland, 1967). Where appropriate, V_{max} values are expressed as micromolar increase in intracellular concentration per minute, assuming an intracellular water volume of 4 ml (g dry wt bacteria)⁻¹ (Kepes & Cohen, 1962).

Fig. 1. Metabolism of (*l*)-5-[Me- ^{14}C]methyl- H_4PteGlu by *Lactobacillus casei*. Bacteria were preincubated for 15 min with (*l*)-5-[Me- ^{14}C]methyl- H_4PteGlu ($0.45\ \mu\text{M}$; sp. act., $47\ \text{mCi mmol}^{-1}$) to an intracellular ^{14}C -labelled vitamin concentration of $99\ \mu\text{M}$, then filtered, washed, and resuspended in fresh buffer and reincubated with shaking, as described in Methods. At 1 (—), 2 (— · —), 5 (·····) and 24 h (---), portions were removed, filtered and extracted with boiling buffer, and intracellular extract (equivalent to $0.8\ \text{mg dry wt bacteria}$) and cell-free medium ($2\ \text{ml}$) were applied to Sephadex G-25 columns ($200 \times 0.7\ \text{cm}$). The columns were eluted with $0.1\ \text{M}$ -phosphate buffer pH 7, containing $0.2\ \text{M}$ -mercaptoethanol, and fractions ($1.7\ \text{ml}$) were collected from the void volume (V_0) as indicated by the elution position of Blue Dextran 2000. The columns were precalibrated with standards whose approximate elution positions were as follows: PteGlu, (fraction 9), 5-methyl- $\text{H}_4\text{PteGlu}_8$ (14), PteGlu $_8$ (16), PteGlu $_4$ (20), PteGlu $_3$ (26), 10-formyl- H_4PteGlu (33), 10-formyl-PteGlu (35), PteGlu $_2$ (35), 5-methyl- H_4PteGlu (49), PteGlu (55), dTTP (16), dTMP (23), ATP (23), methionine (23), formaldehyde (24), formate (24), dT (27), $^3\text{H}_2\text{O}$ (27), *p*-aminobenzoyl-glutamate (28), *S*-adenosylmethionine (31), AMP (37), adenosine (51), adenine (64).

RESULTS

General characteristics of 5-methyl- H_4PteGlu metabolism

After bacteria had been preincubated with labelled 5-methyl- H_4PteGlu for 15 min, more than 95% of the intracellular label chromatographed on Sephadex G-25 at the position of 5-methyl- H_4PteGlu . The initial exit rates of radioactive label after resuspending the bacteria in fresh buffer were 0.01 to $0.02\ \text{min}^{-1}$.

The Sephadex G-25 elution profiles obtained with extracts from bacteria incubated with (*l*)-5-[Me- ^{14}C]methyl- H_4PteGlu showed that within 1 h about 90% of the ^{14}C -label had been transferred to four major metabolites (peaks I, III, IV and V, Fig. 1) together with some high molecular weight material eluting at the void volume. About 20% of the label at 1 h was not extracted from bacteria by boiling buffer. By 2 h, practically no ^{14}C -label in the bacteria or medium eluted at the 5-methyl- H_4PteGlu position (peak VII, Fig. 1). Treatment of the intracellular extracts with conjugase did not affect the Sephadex G-25 elution profiles of ^{14}C -labelled metabolites, indicating that none of the labelled compounds was a folylpolyglutamate. Also, no labelled metabolites comparable to compounds I to VI were found when bacteria were incubated with 5-methyl- H_4 [^3H]PteGlu (Fig. 3), indicating that they were non-folate in nature. This was confirmed by their chromatographic properties on DEAE-cellulose (not shown). With the exception of unmetabolized 5-methyl- H_4PteGlu , none of the labelled compounds chromatographed at the position of

Fig. 2

Fig. 3

Fig. 2. Metabolism of (*dl*)-5-[*Me*- ^{14}C]methyl- H_4PteGlu by *Lactobacillus casei*. Experimental conditions were as described in Fig. 1, except that bacteria were preincubated for 15 min with (*dl*)-5-[*Me*- ^{14}C]methyl- H_4PteGlu ($0.66\ \mu\text{M}$; sp. act., $54\ \text{mCi mmol}^{-1}$) to an intracellular ^{14}C -labelled vitamin concentration of $167\ \mu\text{M}$. The chromatographic patterns of metabolites at 5 (—) and 24 h (---) are shown.

Fig. 3. Metabolism of (*dl*)-5-methyl- $\text{H}_4[^3\text{H}]\text{PteGlu}$ by *Lactobacillus casei*. Experimental conditions were as described in Fig. 1, except that bacteria were preincubated for 15 min with (*dl*)-5-methyl- $\text{H}_4[^3\text{H}]\text{PteGlu}$ ($0.44\ \mu\text{M}$; sp. act., $500\ \text{mCi mmol}^{-1}$) to an intracellular ^{14}C -labelled vitamin concentration of $156\ \mu\text{M}$. The chromatographic patterns of metabolites at 2 (—), 5 (---) and 24 h (.....) are shown. The numbers 1–6 indicate the elution positions of 5-methyl- $\text{H}_4\text{PteGlu}_{1-6}$ ($\text{H}_4\text{PteGlu}_{1-6}$) respectively.

known folate standards. Also, no labelled dTMP, methionine or *S*-adenosylmethionine or its major labelled breakdown product, thiomethyladenosine, was detected.

The metabolism of (*dl*)-5-[*Me*- ^{14}C]methyl- H_4PteGlu was similar to that observed with (*l*)-5-[*Me*- ^{14}C]methyl- H_4PteGlu except that at 5 h, the bacteria and medium still contained a large amount of 5-[*Me*- ^{14}C]methyl- H_4PteGlu which presumably represented the (*d*)-isomer of the vitamin (Fig. 2). This unnatural diastereoisomer is transported at approximately the same rate as the natural (*l*)-isomer (Shane & Stokstad, 1976). In addition, at 5 h the bacteria contained a labelled metabolite (A, Fig. 2) not detected in bacteria preincubated with (*l*)-5-methyl- H_4PteGlu and by 24 h most of the (*d*)-5-methyl- H_4PteGlu had disappeared and another labelled metabolite (B, Fig. 2) had appeared in the bacteria and medium. The elution position of compound B was unaffected by conjugase treatment and it was probably a breakdown product of (*d*)-5-methyl- H_4PteGlu . After conjugase treatment, compound A, which eluted at the position of a pteroyldiglutamate, had identical chromatographic properties to authentic (*d*)-5-methyl- H_4PteGlu both on Sephadex G-25 and DEAE-cellulose. Compound A was thus identified as (*d*)-5-[*Me*- ^{14}C]methyl- $\text{H}_4\text{PteGlu}_2$.

The Sephadex G-25 elution profiles obtained with bacteria preincubated with (*dl*)-5-methyl- $\text{H}_4[^3\text{H}]\text{PteGlu}$ showed that by 2 h there was a variety of ^3H -labelled metabolites (Fig. 3). None of these was similar to metabolites I to VI obtained with (*l*)-5-[*Me*- ^{14}C]methyl- H_4PteGlu (Fig. 1) and no ^3H -labelled material eluted at the void volume. The large 5-methyl- H_4PteGlu peak (Fig. 3) presumably represented the (*d*)-isomer. After conjugase treatment of the intracellular extract, practically all the ^3H -label eluted from DEAE-

cellulose at the position of 5-methyl- H_4 PteGlu and H_4 PteGlu. After 2 h the bacteria contained folates with glutamate chain lengths of up to six, the tetraglutamate (*l*-isomer) and monoglutamate (*d*-isomer) predominating. By 5 h, further chain elongation up to pteroylhexaglutamate had occurred (Fig. 3) and over 80 % of the intracellular 3H -labelled vitamin was identified (after conjugase treatment) as 5-methyl- H_4 PteGlu and H_4 PteGlu. Compound A, observed with bacteria incubated with (*dl*)-5- $[Me-^{14}C]$ methyl- H_4 PteGlu (Fig. 2), was also a labelled metabolite of (*dl*)-5-methyl- $H_4[^3H]$ PteGlu (Fig. 3) and again its chromatographic properties on Sephadex G-25 and DEAE-cellulose, before and after conjugase treatment, indicated that it was (*d*)-5-methyl- H_4 PteGlu₂. By 24 h, most of the intracellular folate had been released into the medium. Intracellular folates had glutamate chain lengths of up to eight and (*d*)-5-methyl- $H_4[^3H]$ PteGlu_{1,2} had disappeared. The chromatographic properties of the two major compounds found in the medium at 2, 5 and 24 h (Fig. 3) were unaffected by conjugase treatment and represented breakdown products, probably 3H -labelled *p*-aminobenzoylglutamate and 3H_2O . The material eluted in the higher polyglutamate region (fractions 5 to 16, Fig. 3), however, was hydrolysed by conjugase to monoglutamate derivatives. Possibly the presence of these polyglutamates in the medium at 24 h reflected losses in cell viability under the conditions of the experiment, rather than release by the bacteria.

Methionine metabolism by L. casei

Although no labelled methionine was detected in bacteria metabolizing 5- $[Me-^{14}C]$ methyl- H_4 PteGlu, methionine is probably rapidly converted to other compounds. To investigate this possibility further, transport and metabolism studies were carried out with L- $[Me-^{14}C]$ -methionine in an analogous manner to those previously described for (*l*)-5-methyl- H_4 PteGlu.

The kinetics of methionine uptake by *L. casei* under these conditions (acetate/phosphate buffer pH 6, containing glucose and mercaptoethanol; see Methods) were best described by two uptake systems with K_m values of 0.14 μM and 150 μM and V_{max} values of 4.0 and 120 $\mu M \text{ min}^{-1}$, respectively. No attempt was made to optimize the conditions for uptake or to ascertain if mercaptoethanol in the buffer had any effect on transport.

If *L. casei* metabolizes 5-methyl- H_4 PteGlu by transfer of the methyl group to homocysteine, then a similar pattern of ^{14}C -labelled metabolites would be expected with $[Me-^{14}C]$ -methionine, as 90 % of the (*l*)-5- $[Me-^{14}C]$ methyl- H_4 PteGlu in preincubated cells was metabolized within 1 h (Fig. 1). However, when bacteria were preincubated with $[Me-^{14}C]$ -methionine to about the same intracellular levels and reincubated in fresh buffer, over 80 % of the intracellular label was not extracted with boiling buffer, compared with about 20 % when bacteria were incubated with ^{14}C -labelled folate.

The Sephadex G-25 elution profiles obtained with extracts from bacteria incubated with L- $[Me-^{14}C]$ methionine (Fig. 4) were very different from the profiles obtained with bacteria metabolizing (*l*)-5- $[Me-^{14}C]$ methyl- H_4 PteGlu (Fig. 1).

Properties of labelled bacterial metabolites not solubilized by buffer extraction

Various treatments were tried to solubilize the ^{14}C -labelled metabolites that were not extracted by boiling buffer from *L. casei* metabolizing (*l*)-5- $[Me-^{14}C]$ methyl- H_4 PteGlu. About 60 % of the label was solubilized after treatment with pancreatic RNAase, about 11 % by DNAase, and the remainder by heating with 1 M-perchloric acid. None of these treatments effectively solubilized ^{14}C -labelled metabolites derived from L- $[Me-^{14}C]$ methionine, suggesting that the metabolism of 5-methyl- H_4 PteGlu proceeded via its oxidation with the incorporation of its one-carbon moiety into purines and pyrimidines rather than via methionine synthesis. To examine this conclusion further, *L. casei* was incubated with labelled purines and pyrimidines, and the metabolites derived from these bases were compared with those derived from 5-methyl- H_4 PteGlu.

Fig. 4

Fig. 5

Fig. 4. Metabolism of L-[Me- ^{14}C]methionine by *Lactobacillus casei*. Experimental conditions were as described in Fig. 1, except that bacteria were preincubated for 15 min with L-[Me- ^{14}C]methionine ($7\text{ }\mu\text{M}$) to an intracellular concentration of $138\text{ }\mu\text{M}$. The chromatographic patterns of ^{14}C -labelled metabolites in the intracellular and extracellular extracts at 1 (—), 2 (····) and 5 h (---) are shown. Standards used to calibrate the column were methionine (A) and $^3\text{H}_2\text{O}$ (B).

Fig. 5. Sephadex G-25 chromatography of labelled metabolites solubilized by RNAase T_2 treatment of insoluble material derived after extraction of *Lactobacillus casei* with boiling buffer. Experimental conditions were as described in Methods. Labelled metabolites solubilized by RNAase treatment of cell debris derived from 10 mg dry wt bacteria were chromatographed on Sephadex G-25 as described in Fig. 1. (a) Patterns of ^{14}C -labelled metabolites derived from (l)-5-[Me- ^{14}C]methyl- H_4PteGlu after incubating bacteria in the presence (---) and absence (—) of unlabelled adenosine (0.1 mM). (b) Patterns of ^3H -labelled metabolites of [^3H]adenine (—) and [^3H]uracil (---). Standards used to calibrate the columns were 3'-UMP (A), $^3\text{H}_2\text{O}$ (B), 3'-AMP (C), 3'-GMP (C) and adenine (D).

Table 1. Distribution of labelled metabolites in *Lactobacillus casei* extracts after incubation with (l)-5-[Me- ^{14}C]methyl- H_4PteGlu and ^3H -labelled bases

Bacteria ($0.2\text{ mg dry wt ml}^{-1}$ in $50\text{ mM-K}_2\text{HPO}_4/100\text{ mM-sodium acetate}$ adjusted to pH 6 with H_3PO_4 , and containing 1% glucose and 5 mM-mercaptoethanol) were preincubated with the ^3H -labelled base ($5\text{ }\mu\text{Ci ml}^{-1}$) for 2 h and with (l)-5-[Me- ^{14}C]methyl- H_4PteGlu ($0.5\text{ }\mu\text{M}$) for 15 min and were then resuspended (0.4 mg ml^{-1}) and reincubated in fresh buffer for 2 h. Extracts were prepared as described in Methods. Results are expressed as pmol labelled compounds ($\text{mg dry wt bacteria}^{-1}$).

Extract	Labelled substrate					
	5-Methyl- $\text{H}_4\text{PteGlu}^*$	Adenine	Guanine	Uracil	Cytosine	Thymine
Intracellular	250	65	531	66	1	107
Insoluble material						
(i) RNAase T_2 extract	25	74	747	72	1	7
(ii) Residue	16	14	108	16	0	405
Medium	250	25	282	29	5	77
Total	544	176	1660	184	8	596

* Values for the labelled metabolites of (l)-5-[Me- ^{14}C]methyl- H_4PteGlu represent the mean of five experiments.

Metabolism of purine and pyrimidine bases

Tritium-labelled bases, with the exception of cytosine, were rapidly taken up by *L. casei*. Initial uptake rates under the conditions described for methionine transport were $10.1 \mu\text{M min}^{-1}$ for adenine (initial concentration in medium $0.27 \mu\text{M}$), $86.8 \mu\text{M min}^{-1}$ for guanine ($1.0 \mu\text{M}$), $13.8 \mu\text{M min}^{-1}$ for uracil ($0.19 \mu\text{M}$), $0.08 \mu\text{M min}^{-1}$ for cytosine ($0.18 \mu\text{M}$), and $10.4 \mu\text{M min}^{-1}$ for thymine ($0.5 \mu\text{M}$). The poor uptake of cytosine was not investigated further but may have been a result of ^3H exchange at the 5-position due to metabolism.

Lactobacillus casei was preincubated for 2 h with the ^3H -labelled bases and for 15 min with (*l*)-5-[$\text{Me-}^{14}\text{C}$]methyl- H_4PteGlu , and was then resuspended in fresh buffer and incubated for 2 h. The distribution of radioactive label in the bacteria and medium at this time is shown in Table 1. Approximately 60% of the non-extractable bacterial ^{14}C -label (insoluble material, Table 1) and over 80% of the adenine, guanine, and uracil metabolites were solubilized by RNAase T_2 , while less than 2% of the thymine metabolites were solubilized by this treatment. When the above experiment was repeated with unlabelled nucleosides (0.1 mM) in the preincubation and resuspension buffers, ^{14}C -label in the RNAase extract was reduced in the presence of adenosine while label in the fraction not solubilized by RNAase was reduced in the presence of thymidine. Uridine had no effect. The insoluble material thus appeared to contain denatured RNA and DNA. This was confirmed by Sephadex G-25 chromatography as ^{14}C -labelled metabolites solubilized by RNAase treatment co-eluted with standard 3'-AMP and 3'-GMP (Fig. 5*a*) and at the same position as the labelled adenine derivative solubilized by this treatment (Fig. 5*b*). The early-eluting labelled adenine metabolite (Fig. 5*b*, fraction 29) was $^3\text{H}_2\text{O}$. Two-dimensional thin-layer chromatography demonstrated that the ^{14}C -label was incorporated into the purine ring. All the ^{14}C -label chromatographed at the positions of 3'-AMP and 3'-GMP and no methylated purine derivatives or 3'-TMP were detected.

The Sephadex G-25 chromatographic profiles obtained with bacterial and extracellular extracts (Table 1) are shown in Fig. 6. The ^{14}C -labelled peak I derived from (*l*)-5-[$\text{Me-}^{14}\text{C}$]methyl- H_4PteGlu coincided with a labelled metabolite derived from uracil (Fig. 6*c*, fraction 18) and thymine (Fig. 6*d*, fraction 18) and eluted just prior to an adenine derivative (Fig. 6*a*, fraction 19). Material in peak I was a thymidylate derivative as a variety of uracil derivatives were detected which were not labelled with ^{14}C (Fig. 6*c*, fractions 10, 14) and its synthesis was decreased when bacteria were preincubated with unlabelled thymidine (0.1 mM) but was unaffected by preincubation with unlabelled uridine (0.1 mM). Peak I co-eluted from Sephadex G-25 with UTP, CTP and dTTP. However, the ^{14}C -labelled compound and [^3H]thymine metabolite in this peak (Fig. 6*d*) eluted from DEAE-cellulose at the position of dTMP (Fig. 7*a*, fraction 15). No [^{14}C]- or [^3H]dTTP was detected. With bacteria incubated with [^3H]uracil, about 90% of the ^3H -label in peak I (Fig. 6*c*) eluted from DEAE-cellulose at the position of dTMP and about 10% at the position of UTP. Peak I therefore contained a thymidylate derivative. It also contained a more electronegative ^{14}C -labelled compound (Fig. 7*a*, fraction 40) which co-eluted with a [^3H]purine derivative (Fig. 6*a*, fraction 19).

The remaining ^{14}C -labelled peaks, with the exception of unmetabolized (*l*)-5-[$\text{Me-}^{14}\text{C}$]methyl- H_4PteGlu , corresponded almost entirely to purine derivatives (Fig. 6*a*, *b*). The major intracellular peak derived from [^3H]adenine was contaminated with $^3\text{H}_2\text{O}$ (Fig. 6*a*, fraction 29). This was not observed in the extracellular extract (Fig. 6*b*) as the medium was concentrated and desalted before chromatography by absorption and elution from active charcoal. ^{14}C -Labelled material not absorbed by charcoal from the medium eluted at the position of formaldehyde (Fig. 6*b*, fraction 28) and condensed with dimedone.

Rechromatography of labelled bacterial compounds in peaks II to V (Fig. 6*a*) on DEAE-cellulose (Fig. 7*b*, *c*, *d*, *e*), in this case with bacteria preincubated with guanine instead of adenine, demonstrated that nearly all the ^{14}C -labelled compounds were purine metabolites.

Fig. 6

Fig. 7

Fig. 6. Metabolism of (*l*)-5-[*Me*- ^{14}C]methyl- H_4PteGlu and ^3H -labelled bases by *Lactobacillus casei*. Experimental conditions were as described in Table 1. Intracellular and extracellular extracts (equivalent to 20 mg dry wt bacteria), obtained by incubating *L. casei* with (*l*)-5-[*Me*- ^{14}C]methyl- H_4PteGlu and ^3H -labelled bases (as indicated), were chromatographed on Sephadex G-25 as described in Fig. 1: ^{14}C -labelled compounds (—); ^3H -labelled compounds (---). Extracellular extracts (b) were concentrated by absorption and elution from active charcoal as described in Methods; the elution pattern of ^{14}C -labelled metabolites not absorbed by charcoal is also shown (.....). The columns were precalibrated with standards whose elution positions were as follows: dTTP (fraction 18), UTP (18), CTP (18), NADP (22), dTMP (25), ATP (26), ADP (32), FAD (34), NAD (34), AMP (41), GMP (41), adenosine (53), adenine (64), formate (25), formaldehyde (26), $^3\text{H}_2\text{O}$ (28), 10-formyl- H_4PteGlu (34) and 5-methyl- H_4PteGlu (51).

Fig. 7. DEAE-cellulose chromatography of labelled metabolites of (*l*)-5-[*Me*- ^{14}C]methyl- H_4PteGlu and ^3H -labelled bases. Experimental conditions were as described in Fig. 6. Individual peak fractions obtained by Sephadex G-25 chromatography of intracellular extracts (Fig. 6) were diluted to 30 ml with H_2O and applied to 25 $\times$ 0.9 cm DEAE-cellulose columns equilibrated with 0.01 M-phosphate buffer pH 6. Each column was eluted by an exponential phosphate gradient formed with 0.01 M-phosphate buffer (100 ml) in a closed mixing chamber attached to a reservoir containing 0.5 M-phosphate buffer pH 6. Fractions (3.0 ml) are numbered from the beginning of the elution: ^{14}C -labelled compounds (—); ^3H -labelled compounds (---). (a) Elution pattern obtained with intracellular labelled material derived from (*l*)-5-[*Me*- ^{14}C]methyl- H_4PteGlu and [^3H]thymine that eluted at the position of peak I from Sephadex G-25 (Fig. 6d, fraction 18). (b–e) Equivalent data for peaks II–V, respectively, obtained from bacteria incubated with (*l*)-5-[*Me*- ^{14}C]methyl- H_4PteGlu and [^3H]guanine (equivalent to Fig. 6a, fractions 23, 27, 33 and 39, respectively). The elution positions of unlabelled standards applied with the samples are indicated on the figures. The elution positions obtained with other standards chromatographed under similar conditions were: dTMP (fraction 15), UMP (16), CMP (16), dTDP (22), UDP (22), CDP (23), AMP (23), GMP (23), dTTP (27), UTP (28), CTP (28), ADP (31), GDP (31), ATP (36), formate (20), 10-formyl- H_4PteGlu (23), 5-methyl- H_4PteGlu (38) and H_4PteGlu (43). Bases, nucleosides, $^3\text{H}_2\text{O}$, formaldehyde, methionine, and *S*-adenosylmethionine eluted at A, which is the elution position of unabsorbed compounds.

Although differences in specific labelling due to different incubation times and differences in initial metabolic routes were observed, practically all the ^3H - and ^{14}C -label co-eluted.

The major purine metabolites in peaks II to IV were not further identified (Fig. 7*b, c, d*). Peak III contained some ATP/GTP (Fig. 7*c*, fraction 39) and labelled material not absorbed by the column consisted of $^3\text{H}_2\text{O}$ and [^{14}C]formaldehyde. Peak IV contained some ADP/GDP (Fig. 7*d*, fraction 31) while most of the labelled material in peak V was identified as AMP/GMP (Fig. 7*e*, fraction 23). The overall distribution of ^{14}C -label in this experiment was as follows: thymidylate derivatives (15%), purine derivatives (43%), formaldehyde (11%), and 5-methyl- H_4PteGlu (31%). No labelled 10-formyl- H_4PteGlu was detected. Practically all the labelled formaldehyde was found in the medium and presumably resulted from degradation of 5,10-methylene- H_4PteGlu under the conditions of this experiment.

DISCUSSION

Although pteroylmono- and pteroyldiglutamates are the forms preferred for transport of folate (Shane & Stokstad, 1975, 1976), in practically every case investigated pteroylpolyglutamates are the predominant, if not the only, intracellular forms of the vitamin (Baugh, Braverman & Nair, 1974; Brown, Davidson & Scott, 1974*a*; Buehring *et al.*, 1974; Houlihan & Scott, 1972; LaVoie *et al.*, 1975; Leslie & Baugh, 1974; Shin, Buehring & Stokstad, 1974*a*; Shin *et al.*, 1972*b*). In *L. casei*, octoglutamates predominate if the organism is grown on low levels of folate (Brown *et al.*, 1974*b*; Buehring *et al.*, 1974); if high levels of folate are supplied in the medium, shorter chain length folates are found, predominantly the tetraglutamate derivative (Baugh *et al.*, 1974). Pteroylpolyglutamates were originally thought to be intracellular storage forms of the vitamin. However, *in vitro* studies have shown them to be equally or more effective than monoglutamates as enzyme substrates. One of these enzymes, a B_{12} -independent methionine synthetase, which is present in some micro-organisms (Taylor & Weissbach, 1973; Whitfield *et al.*, 1970) but not in mammalian tissues (Cheng *et al.*, 1975; Coward *et al.*, 1975), either only functions with folypolyglutamate substrates or utilizes the monoglutamate derivative extremely poorly. *In vivo* studies have also shown that while the rate-limiting step in the utilization of pteroylpolyglutamates by *L. casei* is their transport into the cell, once taken up, they are more effectively utilized for growth than the monoglutamate derivatives (Shane & Stokstad, 1976). The rate-limiting step in the utilization of monoglutamates is some step other than transport.

Lactobacillus casei preincubated with (1)-5-[$\text{Me-}^{14}\text{C}$]methyl- H_4PteGlu metabolized this compound to a variety of labelled metabolites which were non-folate in nature. These were identified as purine and thymidylate derivatives by co-chromatography on Sephadex G-25 and DEAE-cellulose with metabolites derived from [^3H]thymine and [^3H]purines. Delk & Rabinowitz (1975) have shown that folate is involved in the methylation of uridine in RNA of *Streptococcus faecalis*. We did not detect any labelled 3'-TMP in *L. casei* RNA hydrolysates or any methylated purines, demonstrating that the one-carbon moiety of 5-[$\text{Me-}^{14}\text{C}$]methyl- H_4PteGlu was incorporated into the purine ring. This does not rule out the possibility that folate is involved in the methylation of RNA in *L. casei* as our technique was probably not sensitive enough to detect small amounts of labelled methylated bases. In respect to this, we were unable to detect labelled methylated bases in the RNA of cells incubated with [^3H]adenine or [^3H]uracil.

No labelled methionine was detected in these experiments and completely different labelled metabolites were obtained with bacteria incubated with L-[$\text{Me-}^{14}\text{C}$]methionine. These data indicate that the initial step in the metabolism of 5-methyl- H_4PteGlu by *L. casei* proceeds via its oxidation rather than through methionine synthesis. This is different from mammalian metabolism where the one-carbon moiety of 5-methyl- H_4PteGlu is transferred to methionine (Nixon *et al.*, 1973) and oxidation to 5,10-methylene- H_4PteGlu does not occur under physiological conditions (Kutzbach & Stokstad, 1971).

Although no labelled pteroylpolyglutamates were detected in cells incubated with (*l*)-5-[Me-¹⁴C]methyl-H₄PteGlu, (*d*)-5-[Me-¹⁴C]methyl-H₄PteGlu was slowly metabolized to the diglutamate form, but not to higher polyglutamates. In human lymphocytes, however, labelled polyglutamates were not formed from (*dl*)-5-[Me-¹⁴C]methyl-H₄PteGlu (LaVoie, Tripp & Hoffbrand, 1974), although in this case the (*d*)-isomer may not have been transported. (*dl*)-5-Methyl-H₄[³H]PteGlu was metabolized to labelled polyglutamate forms, mainly to (*l*)-5-methyl-H₄PteGlu_{*n*} and H₄PteGlu_{*n*} (*n* ≤ 8) and (*d*)-5-methyl-H₄PteGlu₂, suggesting that (*l*)-5-methyl-H₄PteGlu is not a substrate for the *L. casei* folylpolyglutamate synthetase, and that removal of its one-carbon moiety is necessary before folylpolyglutamates can be synthesized.

Little is known of the specificity of the enzymes responsible for the formation of pteroylpolyglutamates. Sakami *et al.* (1973) reported the presence of two enzymes in *Neurospora crassa*, one specific for the formation of pteroyldiglutamate and the other for synthesis of longer chain polyglutamates and both specific for H₄PteGlu_{*n*} derivatives. *Escherichia coli* contains a 10-formyl-H₄PteGlu₂ synthetase (Masurekar & Brown, 1975) which will also utilize a variety of other pteroylmonoglutamates but not 5-methyl-H₄PteGlu while Gawthorne & Smith (1973), in studies with mixed folate diastereoisomers, reported that 5-formyl-H₄PteGlu, 5-methyl-H₄PteGlu and H₄PteGlu were substrates for the enzyme in sheep liver. Shorter chain length polyglutamates of PteGlu in *L. casei* (Shin, Buehring & Stokstad, 1974*b*) and methotrexate (Baugh, Krumdieck & Nair, 1973; Shin *et al.*, 1974*b*) in rat liver have also been reported. In a previous study (Shane & Stokstad, 1975), we showed that *L. casei* metabolized low levels of PteGlu to a variety of H₄PteGlu polyglutamates. All these data suggest the presence of two synthetases, with the diglutamate-forming enzyme having a specificity for a variety of pteroylmonoglutamates and the higher polyglutamate synthetase having a specificity for H₄PteGlu_{*n*}.

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