Rate-limiting Steps in Folate Metabolism by *Lactobacillus casei*

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(Received 18 May 1977)

Oxidation of 5-methyltetrahydrofolate to 5,10-methylenetetrahydrofolate was the rate-limiting step in 5-methyltetrahydrofolate metabolism by *Lactobacillus casei*. The limiting steps in the utilization of suboptimal levels of folate by *L. casei* were related to the ability of folates to function in purine and/or thymidylate biosynthesis. Folates with glutamate chains of up to at least seven residues were substrates for these biosynthetic enzymes, and comparisons of bacterial growth yields with transport rates for these folates indicated that the polyglutamates were more effective substrates in purine and thymidylate synthesis than the corresponding pteroylmonoglutamates. *Lactobacillus casei* contained low levels of a B₁₂-independent, pteroylpolyglutamate-specific methionine synthetase. Its methylenetetrahydrofolate reductase also functioned more effectively with pteroylpolyglutamate substrates.

INTRODUCTION

The major route for 5-methyltetrahydrofolate (5-methyl-H₄PteGlu) metabolism in *Lactobacillus casei* is oxidation with the incorporation of the one-carbon moiety of 5-methyl-H₄PteGlu into thymidylate and purine derivatives (Shane & Stokstad, 1977).

In this study, the growth requirement of *L. casei* for folates in media containing purines or thymine and the effects of products of one-carbon metabolism on 5-methyl-H₄PteGlu metabolism were investigated to assess rate-limiting steps in folate metabolism.

METHODS

Nomenclature. The abbreviations used are: PteGlu, pteroylglutamic acid, folic acid; PteGlu_n, pteroylmono- to pteroyloligo-γ-L-glutamic acid, *n* indicating the number of glutamic acid residues; H₄PteGlu_n, 5,6,7,8-tetrahydropteroylmono- to 5,6,7,8-tetrahydropteroyloligo-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 (specific activity, 47 mCi mmol⁻¹), (*l*)-10-[formyl-¹⁴C]formyl-H₄PteGlu (sp. act., 47 mCi mmol⁻¹), and PteGlu_n (*n* = 1, 3, 5 and 7) were synthesized and purified as described previously (Shane & Stokstad, 1976). [¹⁴C]Formate (sp. act., 47 mCi mmol⁻¹) was obtained from Schwarz/Mann (Orangeburg, New Jersey, U.S.A.) and unlabelled purines and pyrimidines, nucleosides and amino acids from Sigma.

Organism and growth conditions. *Lactobacillus casei* (ATCC7469) was cultured as described previously (Tamura *et al.*, 1972).

Metabolism of (*l*)-5-[Me-¹⁴C]methyl-H₄PteGlu. *Lactobacillus casei*, harvested from growth media in late exponential phase, was washed with, and resuspended (0.2 mg dry wt ml⁻¹) in 50 mM-K₂HPO₄/100 mM-sodium acetate, adjusted to pH 6 with H₃PO₄, and containing 1% (w/v) glucose and 5 mM-mercaptoethanol. The bacteria were preincubated at 37 °C for 2 h before addition of (*l*)-5-[Me-¹⁴C]methyl-H₄PteGlu (0.45 μM). After a further 15 min, the bacteria were filtered, resuspended in fresh buffer (0.4 mg dry wt ml⁻¹) and re-incubated at 37 °C for 2 h. Intracellular labelled metabolites were extracted with boiling buffer and the incorporation of the labelled one-carbon moiety of 5-methyl-H₄PteGlu into purines, thymidylate and

formaldehyde was determined by Sephadex G-25 and DEAE-cellulose chromatography, as described previously (Shane & Stokstad, 1977). Labelled RNA and DNA in the material not extracted by boiling buffer was determined after treatment with RNAase T₂, as described previously (Shane & Stokstad, 1977). In some cases, the insoluble material was treated with 2% (w/v) HClO₄ for 5 min at 4 °C to extract oligonucleotides, with 10% HClO₄ for 8 h at 20 °C to extract RNA, and with 10% HClO₄ for 2 h at 65 °C to extract DNA (Feinendegen, Bond & Painter, 1961).

Enzyme assays. An enzyme extract was prepared by sonicating *L. casei* (2.7 g dry wt) in 0.1 M-potassium phosphate buffer pH 7 (20 ml) as described by Ohara & Silber (1969). The sonicated extract was centrifuged (10000 g, 15 min) and the supernatant was dialysed against two changes of 0.1 M-potassium phosphate buffer pH 7 (1000 vol.).

B₁₂-dependent methionine synthetase [5-methyltetrahydropteroyl-L-glutamate:L-homocysteine S-methyltransferase; EC 2.1.1.13] and B₁₂-independent methionine synthetase [5-methyltetrahydropteroyl-tri-L-glutamate:L-homocysteine S-methyltransferase; EC 2.1.1.14] were assayed by slightly modified published procedures (Burton & Metzenberg, 1975; Cheng, Shane & Stokstad, 1975; Coward *et al.*, 1975; Kamely, Littlefield & Erbe, 1973; Taylor & Weissbach, 1967) as described in Table 1. L-Homocysteine was prepared from L-homocysteine thiolactone (Duerre & Miller, 1966) just before use. FADH₂ was prepared by reduction of FAD with H₂/PtO₂ (Loughlin, Elford & Buchanan, 1964).

5,10-Methylenetetrahydrofolate reductase [5-methyltetrahydrofolate:NAD⁺ oxidoreductase; EC 1.1.1.68] was assayed in the reverse direction using menadione as an artificial electron acceptor (Cheng *et al.*, 1975; Katzen & Buchanan, 1965; Kutzbach & Stokstad, 1971). The 5,10-[methylene-¹⁴C]methylenetetrahydrofolate product formed, when heated at 95 °C at pH 4.5 in the presence of dimedone, transferred its one-carbon unit to form a formaldehydedimedone adduct which was extracted with toluene. Carrier [¹⁴C]formaldehyde was added to parallel samples to determine recovery (about 63 %).

Treatment of kinetic data. *K_m* and *V_{max}* values were determined by an unweighted non-linear regression method (Wilkinson, 1961).

RESULTS

Methionine synthetase activity

Although the major route of 5-methyl-H₄PteGlu metabolism in *L. casei* does not proceed via methionine synthesis, low levels of 5-methyl-H₄PteGlu-homocysteine methyltransferase were detected in *L. casei* extracts (Table 1). *Escherichia coli* has two methionine synthetases (Taylor & Weissbach, 1973; Whitfield, Steers & Weissbach, 1970). One, a B₁₂-dependent enzyme, will utilize mono- and polyglutamyl methyltetrahydrofolates as substrates, and requires a reducing system and catalytic amounts of S-adenosylmethionine (AdoMet) for activity. The other enzyme is B₁₂-independent, has no AdoMet or reducing system requirement, but requires phosphate and polyglutamyl methyltetrahydrofolates and is stimulated by Mg²⁺. The B₁₂-independent enzyme in *L. casei* extracts was measured by assay system C (Table 1) whereas assay systems A and B also measured the B₁₂-dependent enzyme. In system A, the reducing component was FADH₂ plus H₂ and in B, mercaptoethanol plus cyano-vitamin B₁₂ (Taylor & Weissbach, 1967). Assay B, an aerobic assay for the B₁₂-dependent enzyme, is often used for convenience although it is considerably less active than the anaerobic assay A.

Activities with each methyltetrahydrofolate substrate were similar in all assay systems (Table 1) and no AdoMet requirement was demonstrated. Activity was dependent on the presence of enzyme preparation and homocysteine, and the pentaglutamate substrate was more active than the monoglutamate. These results are consistent with the presence of a B₁₂-independent, polyglutamate-specific methyltransferase. Kinetic constants for the methyltetrahydrofolate substrates are shown in Table 2. Although these were evaluated with crude enzyme preparations, the absence of folyl-γ-glutamyl carboxypeptidase activity in *L. casei* does allow a comparison of folate substrates to be made. At sufficiently low substrate concentrations, the pentaglutamate substrate would be 540 times more effective in methionine synthesis than the monoglutamate.

Table 1. *Requirements for methyl group transfer from methyltetrahydrofolates to homocysteine*

The complete reaction mixtures for assay A contained (in 0.3 ml): (*l*)-5-[$Me-^{14}C$]methyl- H_4 PteGlu (75 μM ; sp. act., 47 mCi mmol⁻¹) or (*l*)-5-[$Me-^{14}C$]methyl- H_4 PteGlu₅ (150 μM ; sp. act., 47 mCi mmol⁻¹), L-homocysteine (500 μM), *S*-adenosylmethionine (250 μM ; AdoMet), cyano-vitamin B₁₂ (50 μM), mercaptoethanol (33 mM), FADH₂ (250 μM), potassium phosphate buffer pH 7 (100 mM) and enzyme extract (1.54 mg protein, prepared as described in the Methods). The mixtures were incubated under H₂ at 37 °C for 60 min and the reactions were terminated by adding ice-cold water (1 ml). [^{14}C]Methionine was separated from labelled folate by chromatography on AG 1-X8 columns (3 × 0.7 cm; Bio-Rad). The columns were washed with water (6.7 ml) and portions (1 ml) of the total effluents (8 ml) were counted.

The complete reaction mixtures for assay B were as in assay A, except that FADH₂ was replaced by mercaptoethanol (200 mM) and the mixtures were incubated aerobically.

The complete reaction mixtures for assay C were as in assay B except that AdoMet and vitamin B₁₂ were replaced by MgCl₂ (100 μM). Mixtures were incubated aerobically.

Blank values were obtained by incubating labelled folates in 0.1 M-potassium phosphate buffer pH 7 under the conditions described for each assay.

Reaction mixture	[^{14}C]Methionine formed [nmol h ⁻¹ (mg protein) ⁻¹] from:	
	(<i>l</i>)-5-[^{14}C]methyl- H_4 PteGlu	(<i>l</i>)-5-[^{14}C]methyl- H_4 PteGlu ₅
Assay A		
Complete	0.23	0.56
– Enzyme	0.01	0.01
– Homocysteine	0.08*	0.02
– AdoMet	0.17	0.57
– Vitamin B ₁₂	0.24	0.60
Assay B		
Complete	0.12	0.75
– Enzyme	0	0
– Homocysteine	0.03	0.04
– AdoMet	0.11	0.79
– Vitamin B ₁₂	0.11	0.76
Assay C		
Complete	0.11	0.76
– Enzyme	0	0
– Homocysteine	0.02	0.03
– MgCl ₂	0.10	0.82

* The small amount of activity in the absence of homocysteine probably represents [^{14}C]thiomethyl-mercaptoethanol.

5,10-Methylenetetrahydrofolate reductase activity

Reductase activity was measured in the reverse direction using menadione as an artificial electron acceptor. Kinetic constants for various (*l*)-5-[$Me-^{14}C$]methyltetrahydrofolate substrates are shown in Table 2. The K_m values decreased with increasing glutamate chain length. No inhibition by AdoMet (1 mM) or *S*-adenosylethionine (1 mM) was observed. When the reaction was carried out for 12 h under a H₂ atmosphere in the absence of menadione, with larger amounts of extract (2 mg protein) and with the various folate substrates, practically no 5,10-methylene- H_4 PteGlu_n was detected suggesting that the equilibrium of the reaction favoured methyltetrahydrofolate synthesis.

Effect of unlabelled analogues on (*l*)-5-[$Me-^{14}C$]methyl- H_4 PteGlu metabolism

To assess which steps in the metabolism of 5-methyl- H_4 PteGlu might be rate-limiting, its metabolism was studied in the presence of a variety of unlabelled analogues. Addition of unlabelled thymidine to the incubation medium decreased the labelled one-carbon incorporation into thymidylate derivatives and DNA but had little effect on labelled one-carbon incorporation into purines (Table 3). Unlabelled adenosine reduced the level of

Table 2. *Kinetic constants of folates for methionine synthetase and 5,10-methylenetetrahydrofolate reductase*

Methionine synthetase activity was measured using assay C (see Table 1) with a 10 min incubation period. 5,10-Methylenetetrahydrofolate reductase activity was assayed as described in Methods. Values for K_m [in μM] and $V_{\max}$ [in $\text{nmol h}^{-1} (\text{mg protein})^{-1}$] are given as average values $\pm$ maximum deviations, with the number of determinations in parentheses.

Folate	Methionine synthetase		5,10-methylenetetrahydrofolate reductase	
	K_m	$V_{\max}$	K_m	$V_{\max}$
(l)-5-methyl- H_4PteGlu	41 ± 17 (7)	0.21 ± 0.03 (7)	9.5 ± 2.5 (10)	6.0 ± 0.5 (10)
(l)-5-methyl- $\text{H}_4\text{PteGlu}_8$	ND	ND	4.8 ± 1.6 (10)	5.4 ± 0.9 (10)
(l)-5-methyl- $\text{H}_4\text{PteGlu}_5$	0.34 ± 0.21 (7)	0.94 ± 0.07 (7)	1.8 ± 0.4 (10)	5.1 ± 0.2 (10)
(l)-5-methyl- $\text{H}_4\text{PteGlu}_7$	ND	ND	1.1 ± 0.3 (8)	4.9 ± 0.4 (8)

ND, Not determined.

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

Experimental conditions were as described in Methods. Bacteria were preincubated in buffer for 2 h, and with (l)-5-[Me- ^{14}C]methyl- H_4PteGlu for 15 min, and then resuspended in fresh buffer and incubated for 2 h. The preincubation and incubation buffers contained the indicated unlabelled additions (1 mM). Results are expressed as pmol ^{14}C -label incorporated (mg dry wt bacteria) $^{-1}$.

Metabolite	Additions to medium						
	None	Adenosine	Thymidine	Thymidine	Formate	Serine	Methionine
Intracellular fraction							
Thymidylate*	42	38	24	14	22	41	39
Purines	65	30	64	27	31	65	68
Formaldehyde	4	3	2	3	3	2	2
5-Methyl- H_4PteGlu	32	22	27	19	7	46	64
Extracellular fraction							
Thymidylate*	17	11	4	4	8	13	11
Purines	127	142	149	154	127	121	110
Formaldehyde	43	91	76	121	168	53	52
5-Methyl- H_4PteGlu	49	40	42	34	28	61	81
RNA fraction							
Purines	25	5	19	7	12	24	22
DNA fraction							
Thymidylate*	17	14	4	3	5	12	17
Total	423	396	417	387	414	445	472

* Thymidylate derivatives.

labelled purines and RNA in the bacteria but increased the labelled purines in the medium. As the total synthesis of labelled purines was not significantly affected by adenosine, its major effect was probably to inhibit the re-uptake of labelled purines released by the bacteria. Labelled 10-formyl- H_4PteGlu was not detected. Thymidine and adenosine also increased the level of labelled formaldehyde in the medium at the expense of 5-[Me- ^{14}C]methyl- H_4PteGlu . Serine, methionine and uridine (not shown) had no significant effect on the distribution of labelled metabolites. Addition of formate decreased labelled one-carbon incorporation into purines and thymidylate derivatives. Labelled 10-formyl- H_4PteGlu was not detected. A large increase in labelled formaldehyde in the medium was observed and the total level of 5-[Me- ^{14}C]methyl- H_4PteGlu was reduced. The major effect of formate was either to increase the pool of 5,10-methylene- H_4PteGlu with a consequent dilution of the radioactive label or to disturb the equilibrium between 5,10-methylene- H_4PteGlu and

Table 4. *Distribution of labelled metabolites in Lactobacillus casei after incubation with labelled formate or folates*

Experimental conditions are described in Methods. Bacteria were preincubated for 15 min in buffer containing the indicated additions and then resuspended in fresh buffer and incubated for 1 h.

Additions to preincubation buffer	¹⁴ C-label incorporated [pmol (mg dry wt bacteria) ⁻¹] into:						
	Thymidylate	Purines	Formate	Formaldehyde	RNA	DNA	5-methyl-H ₄ PteGlu Total†
[¹⁴ C]Formate (1 μM)							
+ H ₄ PteGlu (0.5 μM)	112	403	0	23	357	101	0 1070
[¹⁴ C]Formate (1 mM)*							
+ H ₄ PteGlu (0.5 μM)	5610	11800	2.7 × 10 ⁶	4500	13600	2980	0 2.8 × 10 ⁶
[¹⁴ C]Formate (1 μM)							
+ 5-methyl-H ₄ PteGlu (0.5 μM)	92	280	0	10	304	97	0 842
[¹⁴ C]Formate (1 mM)*							
+ 5-methyl-H ₄ PteGlu (0.5 μM)	3860	8700	2.8 × 10 ⁶	3740	11000	2770	0 2.8 × 10 ⁶
(l)-10-[¹⁴ C]formyl-H ₄ PteGlu (0.5 μM)	43	349	0	1	293	49	0 752
(l)-5-[¹⁴ C]methyl-H ₄ PteGlu (0.5 μM)	22	90	0	10	25	12	340 520
³ H-label incorporated [pmol (mg dry wt bacteria) ⁻¹] into:							
	5-methyl-H ₄ PteGlu	10-formyl-H ₄ PteGlu	H ₄ PteGlu	H ₂ PteGlu	PteGlu		
(l)-H ₄ [³ H]PteGlu (0.5 μM)	34	9	204	106	189		
(l)-5-methyl-H ₄ [³ H]PteGlu (0.5 μM)	310	11	289	45	99		

* 1 mM-[¹⁴C]formate was also added to the resuspension buffer.

† In some cases, not all of the labelled metabolites were identified.

formaldehyde by removing free H₄PteGlu. To investigate whether formate was metabolized to 5,10-methylene-H₄PteGlu, *L. casei* was preincubated with [¹⁴C]formate and its labelled metabolites were identified.

Metabolism of labelled formate and (l)-10-formyl-H₄PteGlu

The labelled one-carbon of [¹⁴C]formate was rapidly incorporated into RNA and DNA (Table 4). The formation of labelled formaldehyde and thymidylate and purine derivatives demonstrated that formate metabolism proceeded via 10-formyl-H₄PteGlu and 5,10-methylene-H₄PteGlu. However, no labelled 10-formyl-H₄PteGlu or 5-methyl-H₄PteGlu was detected, even when *L. casei* was incubated with 1 mM-[¹⁴C]formate. Under these conditions there was an increased incorporation of radioactive label into RNA, DNA, thymidylate, purines and formaldehyde suggesting that the rate-limiting step in formate metabolism was the synthesis of 10-formyl-H₄PteGlu. Identical labelled metabolites were found after incubating *L. casei* with (l)-10-[formyl-¹⁴C]formyl-H₄PteGlu (Table 4). Within 1 h, the labelled one-carbon had been incorporated entirely into RNA, DNA, thymidylate and purine derivatives and no labelled folates were detected.

Similar labelled metabolites were found after incubating *L. casei* with (l)-5-[Me-¹⁴C]-methyl-H₄PteGlu (Table 4). However, the incorporation of the labelled one-carbon into thymidylate and purines proceeded at a much slower rate than was observed with formate and 10-formyl-H₄PteGlu and, after the 1 h incubation, much of the 5-methyl-H₄PteGlu remained unmetabolized. The rate-limiting step in the metabolism of 5-methyl-H₄PteGlu was oxidation to 5,10-methylene-H₄PteGlu.

This pattern of metabolism was reflected by the labelled folates found after incubating

Table 5. *Relative yields of Lactobacillus casei in response to folates, purines and thymine*

Bacterial growth in the presence of the indicated compounds was measured as described by Tamura *et al.* (1972). Growth responses were assessed using suboptimal concentrations of the compounds (i.e. where cell yield was proportional to the extracellular compound concentration) and is expressed relative to PteGlu (= 100).

Compound tested	Relative cell yield per nmol in medium* containing:		
	Purines	Thymine	No addition
PteGlu†	100	100	100
PteGlu ₃	91	107	93
PteGlu ₅	20	25	26
PteGlu ₇	2.2	2.2	1.8
(l)-5-Methyl-H ₄ PteGlu	115	106	100
(l)-5-Methyl-H ₄ PteGlu ₃	91	93	89
(l)-5-Methyl-H ₄ PteGlu ₅	24	25	23
(l)-5-Methyl-H ₄ PteGlu ₇	0.15	0.23	0.28
Thymine	0.004		
Adenine		0.0008	
Guanine		0.0007	

* The medium was a complex mixture (Tamura *et al.*, 1972) containing amongst other compounds: serine (3 mM), glutamate (9 mM), glycine (1.3 mM), methionine (1.1 mM) and uracil (45 μ M). Purines (136 μ M) [adenine, 37 μ M; guanine, 33 μ M; xanthine, 66 μ M] and thymine (90 μ M) were added to make media containing purines and thymine respectively.

† The cell yield per nmol obtained with suboptimal levels of PteGlu in the medium corresponded to: $E_{640}^{1\text{cm}} = 296$ for medium containing purines; $E_{640}^{1\text{cm}} = 124$ for medium containing thymine; and $E_{640}^{1\text{cm}} = 133$ for medium without additions.

L. casei with (l)-H₄[³H]PteGlu and (l)-5-methyl-H₄[³H]PteGlu (Table 4). After the 1 h incubation, H₄PteGlu remained unchanged or had been metabolized mainly to H₂PteGlu or PteGlu. Very little 5-methyl-H₄PteGlu or 10-formyl-H₄PteGlu was formed. The patterns of labelling of the folate metabolites derived from 5-methyl-H₄PteGlu were similar apart from the presence of unmetabolized 5-methyl-H₄PteGlu.

Lactobacillus casei growth response to folates, purines and thymine

Previous studies have shown that purines and thymine will partially replace the folate requirement of *L. casei* (Rogers & Shive, 1948; Stokstad, 1941). We confirmed these observations and found that adenine (1 mM) plus thymine (10 μ M) was about 80% as effective as excess folate in promoting growth of this micro-organism. Additional thymine had no effect while additional purine was not investigated due to problems with solubility. Half-maximum cell yield was achieved with 1.8 μ M-thymine (purine in excess) and with 46 μ M-adenine (thymine in excess). Adenosine did not replace adenine as a growth promoter. Uracil was essential for growth in the presence of folates.

As *L. casei* does not possess any γ -glutamylcarboxypeptidase activity (Buehring, Tamura & Stokstad, 1974; Shane & Stokstad, 1975), a comparison of bacterial growth on various pteroylpolylglutamates with either purine or thymine limiting should indicate whether the enzymes responsible for the synthesis of these compounds respond to the same polyglutamate chain lengths. The relative response of *L. casei* to a variety of folates remained the same, regardless of whether purine, thymine, or both were limiting (Table 5). Furthermore, folates with glutamate chain lengths of at least up to seven served as cofactors in purine and thymidylate biosynthesis. Comparisons of the transport rates of these folates with the ability of the folates to promote growth of *L. casei* (Shane & Stokstad, 1976) have shown that the longer chain length polyglutamates are more effectively utilized by the bacteria, once taken up, than the monoglutamates. The response to 5-methyl-H₄PteGlu₇

was very poor and, at high concentrations of this compound, the maximum cell yield observed was considerably lower than those obtained with all the other folates tested. The poor cell yield with this compound was almost certainly due to its poor transport into the cell, and was not a result of metabolism, as PteGlu₅ was effective in intracellular metabolism. We have previously shown that 5-methyl-H₄PteGlu₅ is poorly transported by *L. casei* compared with PteGlu₅ (Shane & Stokstad, 1976) although, as the relative cell yield data (Table 5) indicate, it is as active as a growth promoter, since it is more effective than PteGlu₅ in intracellular metabolism.

The growth response to folates in the medium with no additions and in the medium containing excess thymine were almost identical (Table 5) indicating that purine biosynthesis was rate-limiting in both cases. The growth response to folates was 2.3-fold higher in medium containing purines, also indicating that less folate was required to meet the organism's thymidylate requirements than its purine requirements. The differences in folate requirement for thymidylate and purine biosynthesis were probably greater than these results suggest, as the purine (136 μ M) in the medium containing purine was not in excess and some folate was probably utilized for purine biosynthesis. This medium is the standard medium used for the assay of folates in biological samples (Tamura *et al.*, 1972) and contains sufficient purine to prevent spurious results caused by contaminating purines in these samples.

DISCUSSION

Although other workers could not detect any methionine synthetase activity in *L. casei* (Galivan, 1971; Kisiuk, 1971), low levels of the enzyme were detected using labelled substrates of high specific activity. The properties of the synthetase were similar to those reported for the polyglutamate-specific, B₁₂-independent enzyme from *E. coli* (Taylor & Weissbach, 1973; Whitfield *et al.*, 1970). (*l*)-5-Methyl-H₄PteGlu₅ was more than 500 times as effective a substrate as (*l*)-5-methyl-H₄PteGlu at low substrate levels. No B₁₂-dependent transmethylase was detected although the apoenzyme may have been present, as vitamin B₁₂ was not added to the culture medium. The specific activity of the transmethylase was 2 to 5 orders of magnitude lower than that of thymidylate synthetase (Crusberg, Leary & Kisiuk, 1970), dihydrofolate reductase, 10-formyltetrahydrofolate synthetase, and 5,10-methylenetetrahydrofolate dehydrogenase (Ohara & Silber, 1969; Silber & Mansouri, 1971) in extracts of *L. casei*.

The 5,10-methylenetetrahydrofolate reductase of *L. casei* also functioned more effectively with polyglutamyl folate substrates. It was not inhibited by AdoMet which is a non-competitive inhibitor of the mammalian reductase (Kutzbach & Stokstad, 1967). However, the equilibrium of the reaction, which favours 5-methyl-H₄PteGlu_n formation, was similar to that observed with the mammalian and *Escherichia coli* reductases (Cheng *et al.*, 1975; Katzen & Buchanan, 1965; Kutzbach & Stokstad, 1971). Because of this equilibrium, the reductase reaction has often been considered to be essentially irreversible. The reaction is normally assayed in the reverse direction using menadione as an artificial electron acceptor to drive the oxidation of 5-methyl-H₄PteGlu_n to near completion. However, menadione does not affect the reaction rate for the oxidation of 5-methyl-H₄PteGlu_n, it only prevents the 5,10-methylene-H₄PteGlu_n formed from being reduced back to 5-methyl-H₄PteGlu_n. The kinetic constants obtained for the *L. casei* reductase adequately accounted for the observed metabolism of 5-[Me-¹⁴C]methyl-H₄PteGlu by this organism and the consequent incorporation of the labelled one-carbon moiety into purine and thymidylate derivatives (Shane & Stokstad, 1977). The *L. casei* auxotrophic requirement for purines and thymine has been known for some time (Rogers & Shive, 1948; Stokstad, 1941). However, previously it had been assumed that 5-methyl-H₄PteGlu was metabolized via methionine synthesis with the consequent release of H₄PteGlu which could then be utilized for biosynthesis of these compounds.

Although the equilibrium of the 5,10-methylenetetrahydrofolate reductase reaction appeared to favour the reduction of 5,10-methylene- H_4 PteGlu, the labelled one-carbon of [^{14}C]formate was rapidly incorporated into purines and thymidylate via 10-formyl- and 5,10-methylene- H_4 PteGlu, and not into 5-methyl- H_4 PteGlu. Some reduction of 5,10-methylene- H_4 PteGlu did occur as evidenced by the metabolism of a small proportion of H_4 [^3H]PteGlu to 5-methyl- H_4 [^3H]PteGlu and the increased net metabolism of 5-[Me - ^{14}C]-methyl- H_4 PteGlu when the 5,10-methylene- H_4 PteGlu pool was expanded after incubating *L. casei* with 1 mM-formate. However, the rapid incorporation of the one-carbon moiety of 5,10-methylene- H_4 PteGlu into thymidylate and purines, which is consistent with the relative high levels of thymidylate synthetase and 5,10-methylenetetrahydrofolate dehydrogenase in this bacterium, prevented its reduction back to 5-methyl- H_4 PteGlu. Oxidation of 5-methyl- H_4 PteGlu by 5,10-methylenetetrahydrofolate reductase was the rate-limiting step in the metabolism of 5-methyl- H_4 PteGlu.

10-[formyl- ^{14}C]Formyl- H_4 PteGlu and [^{14}C]formate were rapidly metabolized by *L. casei* with incorporation of the labelled one-carbon moiety into thymidylate and purine derivatives. No labelled 10-formyl- H_4 PteGlu was detected after incubating bacteria with 1 mM-[^{14}C]formate indicating that the 10-formyltetrahydrofolate synthetase reaction was rate-limiting in formate metabolism. Although it is possible that some [^{14}C]formate was metabolized to [^{14}C]purines by non-folate-dependent reactions such as $^{14}CO_2$ -fixation, we consider such a mechanism unlikely or, at most, to be responsible for only a small proportion of the labelled purine formed. It should be noted that the thymidylate formed from [^{14}C]formate or 5-[Me - ^{14}C]methyl- H_4 PteGlu was labelled in the 5-methyl group and not in the pyrimidine ring, demonstrating its formation via 5,10-[methylene- ^{14}C]methylene- H_4 PteGlu.

In evaluating intracellular effectiveness of mono- and polyglutamyl folates by comparing growth enhancement with transport rates (Shane & Stokstad, 1976), two factors should be borne in mind. One is that if a polyglutamate is more effective than a monoglutamate as an enzyme substrate, this increased effectiveness will tend to be magnified due to the exponential growth of the bacteria. However, as the monoglutamate can be metabolized by the micro-organism to polyglutamyl forms, this will tend to have the opposite effect, decreasing the differences between the two forms. Fortunately, *L. casei* has no conjugase enzyme and breakdown of polyglutamates to monoglutamates does not complicate the picture further. Obviously, an exact comparison of various folates cannot be achieved by this method, but a relative comparison can be made.

Comparisons of the transport rates for various folates with their abilities to support growth of *L. casei* (Shane & Stokstad, 1975, 1976) demonstrated that intracellular polyglutamates were more effective in promoting growth than monoglutamates. This was true regardless of whether purine or thymine was limiting in the medium, although less folate was required to meet the organism's thymidylate requirement than was needed for purine biosynthesis. Polyglutamates of chain length at least up to seven served as cofactors in both purine and thymidylate biosynthesis and no selectivity existed between glutamate chain length requirements for purine biosynthesis compared with thymidylate biosynthesis.

The rate-limiting steps in the intracellular utilization of folates by *L. casei* are obviously related to the ability of folates to act as cofactors in purine and thymidylate biosynthesis. At suboptimal folate concentrations, 5-methyl- H_4 PteGlu_n was about equally as effective in promoting growth of *L. casei* as PteGlu_n so oxidation of 5-methyl- H_4 PteGlu does not appear to be limiting in the utilization of folates under these conditions. The most likely rate-limiting steps are the purine and thymidylate biosynthetic reactions themselves. The actual flux through these biosynthetic pathways at limiting folate concentrations will depend on the K_m values of the folate derivatives for the biosynthetic enzymes. The greater growth-promoting abilities of intracellular polyglutamates compared with monoglutamates implies that these polyglutamates have a lower K_m value for the purine and

thymidylate biosynthetic enzymes than the monoglutamates. While this has not been shown by *in vitro* studies for the purine biosynthetic enzymes, Kisliuk, Gaumont & Baugh (1974) have demonstrated that *L. casei* thymidylate synthetase functions more effectively with the hexaglutamate substrate than it does with the corresponding monoglutamate. In addition, polyglutamates such as 5-methyl- $H_4PteGlu_n$ can function in methionine synthesis with the consequent formation of $H_4PteGlu_n$, which can then be utilized for purine and thymidylate synthesis.

Conversion of monoglutamates to polyglutamyl forms cannot be considered rate-limiting in the growth of *L. casei* on monoglutamates since monoglutamates will function in purine and thymidylate biosynthesis. In our experiments, methionine (at about 1 mM) was supplied in the growth medium. However, if methionine is omitted from the growth medium, the pteroylpolyglutamate synthetase reaction would probably become the rate-limiting step in the utilization of monoglutamates.

This research was supported by United States Public Health Service Grant AM-08171 from the National Institutes of Health. B.S. is indebted to the Wellcome Trust for a travel grant. We would like to thank Joe Watson for his excellent technical assistance.

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