The Microbial Biosynthesis of Methionine

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1. The enzymes leading to the methylation of homocysteine have been examined in three micro-organisms: a cobalamin-producing bacterium, *Bacillus megaterium*; a yeast, *Candida utilis*; and a basidiomycete fungus, *Coprinus lagopus*. The yeast and the fungus contain negligible endogenous cobalamin. 2. Extracts of each organism catalyse C₁-transfer from serine to homocysteine with a polyglutamate folate coenzyme. 3. The enzymes generating the methyl group of methionine from C-3 of serine have similar properties in each case, but different mechanisms of homocysteine transmethylation from 5-methyltetrahydrofolates were found. 4. *B. megaterium* contains an enzyme with properties suggestive of a vitamin B₁₂-dependent homocysteine transmethylation, whereas *Cand. utilis* and *Cop. lagopus* transfer the methyl group by a reaction characteristic of the cobalamin-independent mechanism established for *Escherichia coli*. 5. The specificity of each transmethylation for a 5-methyltetrahydropteroylpolyglutamate is consistent with the results of analyses of endogenous folates in these organisms, which showed only conjugated forms. 6. None of the extracts catalysed methionine production from S-adenosylmethionine and homocysteine. 7. These results are compared with results now available for methionine synthesis in other organisms, which show a considerable diversity of mechanisms.

Folic acid derivatives† participate universally in many metabolic reactions involving C₁-units at various levels of oxidation. Vitamin B₁₂, although important to mammalian metabolism, is probably exclusively microbial in origin (Smith, 1951), and many cobamide derivatives have been isolated, e.g. from *Propionibacterium*, species of which synthesize the vitamin in great excess. It is in the methyl-transfer reactions of methionine synthesis, methane formation and acetate production that established pathways involving cobalamin and folates converge directly. The general importance of methionine and the indispensability of folate to cellular biochemistry contrast with the probable participation of vitamin B₁₂ in the metabolism of relatively few micro-organisms; this encouraged further study of the inter-relationships between the two vitamins in the methylation of homocysteine.

Detailed studies of methionine biosynthesis in two

bacteria, *Escherichia coli* and *Bacillus subtilis*, which neither synthesize nor require an exogenous supply of cobalamin, have been reported (Woods *et al.*, 1965; Salem *et al.*, 1972). *E. coli* has been shown to possess alternative means of converting homocysteine into methionine; the two mechanisms differ in their folate specificity and their requirement for cobalamin; *B. subtilis* has only a transmethylation independent of vitamin B₁₂. In the present paper, we examine a cobalamin-producing bacterium, *Bacillus megaterium*, a yeast, *Candida utilis*, and a basidiomycete fungus, *Coprinus lagopus*, for the occurrence of alternative pathways to methionine. The results are discussed with reference to available results for other organisms.

Materials and Methods

Organisms

Stock cultures of *B. megaterium* (N.C.I.B. 8508) and *Cop. lagopus*, strain H5 (Lewis, 1961), were maintained on slopes of nutrient agar (Oxoid CM3), subcultured monthly and stored at 4°C. *Cand. utilis* (N.C.Y.C. 321) was maintained on a medium containing (w/v): malt extract, 0.6%; yeast extract, 0.3%; peptone (Oxoid L37), 0.5%; glucose, 0.5%; agar, 2%.

Escherichia coli strains 3/62 and 121/176 were maintained, cultured and extracted as described by Salem *et al.* (1972).

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† Abbreviations: for members of the folic acid group, based on PteGlu₁ for pteroylglutamate: H₄PteGlu₁, tetrahydropteroylmonoglutamate; H₄PteGlu₃, tetrahydropteroyltriglutamate etc.; derivatives of these compounds are indicated as, e.g. 5-CH₃-H₄PteGlu₃ (the term folate is used in this paper in reference to both simple and conjugated members of the folic acid family of vitamins).

Media and growth

B. megaterium was grown in liquid culture in a glucose-salts medium containing CoCl_2 , and supplemented with yeast extract, 0.2% (Duff & Webley, 1958). For *Cand. utilis*, the minimal medium contained per litre (pH 6.8): KH_2PO_4 , 2.0 g; K_2HPO_4 , 1.5 g; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; glucose, 2.0 g. For each organism, Erlenmeyer flasks (5 litres) containing 1 litre of growth medium were inoculated with about 3×10^8 cells of an 18 h culture in the same medium and shaken at 30°C. Growth of *B. megaterium* was assessed at 650 nm and that of *Cand. utilis* at 450 nm.

Cop. lagopus was grown in a glucose-salts medium containing per litre (pH 6.8): Na_2HPO_4 , 1.8 g; KH_2PO_4 , 0.7 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12 g; NH_4Cl , 0.5 g; glucose, 10 g; and supplemented with thiamin hydrochloride, 40 µg. Erlenmeyer flasks (1 litre) containing 400 ml of medium were inoculated with a mycelial inoculum obtained from a fresh culture on solid medium, and were shaken at 37°C for 40 h. After this period, the yield was 5–8 g wet wt. of mycelium/litre. The yield at end-growth was about 15 g wet wt./litre.

For media supplemented with cobalamin (5 µg/litre), the vitamin was added as a sterile solution to the autoclaved medium.

Cell-free extracts

Ultrasonic extracts of bacteria were prepared and stored as described by Cauthen *et al.* (1966).

For *Cand. utilis*, the freshly harvested organism was suspended in 20 mM-potassium phosphate buffer, pH 7.8, containing 2-mercaptoethanol (7 mM), and was disrupted by two passages through a French pressure cell at a pressure of 7800 lb/in² (53.78 MN/m²) (French & Milner, 1955).

For *Cop. lagopus*, the washed mycelium was homogenized in a buffer (4 ml/g wet wt. of organisms) containing tris-HCl, pH 7.8 (50 mM), 2-mercaptoethanol (10 mM) and glycerol (10%, v/v), by using a glass Potter-Elvehjem homogenizer. About 30 strokes were necessary to disperse visible aggregates of mycelium.

In each case, the crude extracts were centrifuged (25000g; 20 min) and the supernatant fluids were retained as a source of enzymes. The protein content was determined spectrophotometrically by the method of Layne (1957).

The methods for the isolation, chromatography and determination of cobalamin, tests for enzymes concerned in methionine synthesis and the degradation of *S*-adenosylmethionine are described by Salem *et al.* (1972), who also give details of the determination and chromatography of folates isolated from micro-organisms. The sources of chemicals are listed in the same paper.

Results and Discussion

Present knowledge about the methylation of homocysteine to form methionine in micro-organisms stems mainly from investigations with *E. coli* (see Blakley, 1969). The methyl group of methionine may be derived from serine (or glycine) via 5,10-methyl-ene-tetrahydrofolate. Methyl transfer from a conjugated 5-methyltetrahydrofolate to homocysteine is catalysed by a transmethyase requiring only Mg^{2+} (or Mn^{2+}) ions and orthophosphate as cofactors (Guest *et al.*, 1964b; Whitfield *et al.*, 1970). An alternative reaction, involving a cobamide enzyme, arises only after inclusion of cobalamin in the growth medium or after addition of cobalamin to incubation mixtures containing ultrasonic extracts. In the cobalamin-dependent transmethylation, either 5- $\text{CH}_3\text{-H}_4\text{PteGlu}_3$ or 5- $\text{CH}_3\text{-H}_4\text{PteGlu}_1$ may donate the methyl group to homocysteine (Guest *et al.*, 1964b). *E. coli*, grown in the absence of added cobalamin, contains negligible amounts of the vitamin (Woods *et al.*, 1965), and mutant strains lacking the vitamin B₁₂-independent transmethyase require the addition of either methionine or cobalamin for growth (Guest *et al.*, 1964b).

The cobalamin content of each organism used in this study was determined by using *E. coli* 113/3, as described by Foster *et al.* (1964), after removal of methionine with Dowex 1 resin (X8; OH⁻ form; 100–200 mesh). *B. megaterium*, an over-producer of cobalamin (Garibaldi *et al.*, 1953; Perlman, 1959), contained 21 200 ng of cobalamin/g dry wt. of organisms, after growth to stationary phase (1.20 mg dry wt. of cells/ml) and the supernatant medium contained 200 ng of cobalamin/ml. However, *Cand. utilis* (12 ng of cobalamin/g dry wt. of organisms) and *Cop. lagopus* (0.3 ng/g dry wt. of organisms) were similar to *E. coli* (3 ng/g dry wt. of organism; Cauthen *et al.*, 1966) and contained only traces of the vitamin. With the exception of *B. megaterium*, the apparent cobalamin content of these organisms is probably insignificant, for *Salmonella typhimurium* contains 700–1100 ng of cobalamin/g dry wt. of organisms, yet this is insufficient to permit growth of cobalamin/methionine auxotrophs that have lost the cobalamin-independent homocysteine transmethyase by mutation (Cauthen *et al.*, 1966). Subsequent tests for the vitamin B₁₂-dependent methylation of homocysteine in *Cand. utilis* and *Cop. lagopus* were performed with organisms grown with an exogenous supply of cyanocobalamin (5 µg/litre).

Overall conversion of serine and homocysteine into methionine

The methylation of homocysteine with serine as C₁-source was catalysed by Dowex-treated extracts of *B. megaterium*, *Cand. utilis* and *Cop. lagopus*

Table 1. 5,10-Methylenetetrahydrofolate reductase and 5-methyltetrahydrofolate-homocysteine transmethylation in *B. megaterium*, *Cand. utilis* and *Cop. lagopus*

Ultrasonic extracts, each containing 3 mg of protein, were incubated in a mixture containing serine and homocysteine (mixture M1 of Salem *et al.*, 1972). Incubations were for 1 h at 37°C under H₂. All extracts were first passed through columns of Dowex 1 resin (Cl⁻ form) to remove endogenous folates. *E. coli* 3/62 was grown with both DL-methionine and cyanocobalamin; *E. coli* 121/176 was supplemented with DL-methionine only.

Extracts	Source of folate ...	L-Methionine formed (nmol)	
		H ₄ PteGlu ₁	H ₄ PteGlu ₃
<i>B. megaterium</i>		5	36
<i>Cand. utilis</i>		5	57
<i>Cop. lagopus</i>		5	38
<i>E. coli</i> 3/62		2	8
<i>E. coli</i> 3/62 + <i>B. megaterium</i>		58	62
<i>E. coli</i> 3/62 + <i>Cand. utilis</i>		39	78
<i>E. coli</i> 3/62 + <i>Cop. lagopus</i>		50	61
<i>E. coli</i> 121/176		5	11
<i>E. coli</i> 121/176 + <i>Cand. utilis</i>		3	187
<i>E. coli</i> 121/176 + <i>Cop. lagopus</i>		15	61

when H₄PteGlu₃ was the added folate coenzyme (Table 1). The relative inactivity of H₄PteGlu₁ suggests that at least one reaction in methionine synthesis is specific for a polyglutamate folate. This specificity was unaltered by growth of *Cand. utilis* or *Cop. lagopus* with added cobalamin before extraction.

The occurrence of 5,10-methylenetetrahydrofolate reductase and 5-methyltetrahydrofolate-homocysteine transmethylation activities in extracts of the test organisms was investigated by incubation with extracts of *E. coli* 3/62 and *E. coli* 121/176 respectively, together with the substrates and likely cofactors for the conversion of serine and homocysteine into methionine. Extracts of all three test organisms stimulated the production of methionine by an extract of *E. coli* 3/62, which lacks 5,10-methylenetetrahydrofolate reductase (Guest *et al.*, 1964a), when the folate supplied was H₄PteGlu₃ or H₄PteGlu₁ (Table 1), implying the common occurrence of this enzyme. With *Cand. utilis*, there was a marked specificity for a polyglutamate folate cofactor.

Extracts of *E. coli* 121/176 grown in minimal medium supplemented only with methionine are deficient in the cobalamin-independent homocysteine transmethylation (Guest *et al.*, 1964b), whereas the vitamin B₁₂-dependent transmethylation is present only as apoenzyme. A 5-methyltetrahydrofolate-homocysteine transmethylation in extracts of *Cand. utilis* and *Cop. lagopus* was suggested by their ability to stimulate methionine synthesis by *E. coli* 121/176 extract (Table 1). This activity was, however, specific for

a polyglutamate folate. (The high cobalamin content of *B. megaterium* precluded assay of 5-methyltetrahydrofolate-homocysteine transmethylation by this method.)

Use of methyltetrahydrofolates for transmethylation

The transmethylation activity in extracts was explored further by the use of 5-[¹⁴C]methyltetrahydrofolates as methyl donors to homocysteine (Table 2). Extracts of *Cop. lagopus* grown with or without added vitamin B₁₂ appeared to contain a cobalamin-independent transmethylation only. The activity with 5-CH₃-H₄PteGlu₃ was not increased, nor was transmethylation from 5-CH₃-H₄-PteGlu₁ initiated, by incubation under H₂, even when the cofactors *S*-adenosylmethionine, methylcobalamin and the reducing system (H), were added (Table 2a).

A similar situation was found with extracts of *Cand. utilis* grown in minimal medium (Table 2b), for which anaerobic incubation did not significantly increase transmethylation from 5-CH₃-H₄PteGlu₃ even with the added cofactors required by *E. coli* cobalamin-dependent transmethylation. Growth of *Cand. utilis* with added cyanocobalamin, however, apparently changed the properties of its homocysteine transmethylation(s). The 5-CH₃-H₄PteGlu₁ could now serve as a methyl donor to homocysteine to a limited extent, but only under anaerobic conditions. Further, although the ability to methylate homocysteine aerobically with 5-CH₃-H₄PteGlu₃ was not significantly affected by growth of *Cand. utilis* with vitamin B₁₂,

Table 2. 5-[^{14}C]Methyltetrahydrofolate-homocysteine transmethylase in extracts

Extracts (3–10 mg of protein/ml) were incubated at 37°C in a mixture containing DL-homocysteine (22 mM) and MgSO_4 (4.5 mM) together with either 5- $^{14}\text{CH}_3\text{-H}_4\text{PteGlu}_1$ or 5- $^{14}\text{CH}_3\text{-H}_4\text{PteGlu}_3$ (0.3–3 mM; 0.5 $\mu\text{Ci}/\mu\text{mol}$). Other additions where shown were: *S*-adenosylhomocysteine (10 mM, replacing homocysteine); *S*-adenosylmethionine (20 μM); methylcobalamin (10 μM); ($\pm$) $\text{H}_4\text{PteGlu}_3$ (6 mM); reducing system (H), containing FAD (75 nmol), NAD (250 nmol), ethanol (50 μmol) and yeast alcohol dehydrogenase (90 μg). Tubes were incubated for 1 h aerobically with shaking or in an atmosphere of H_2 . The formation of [$\text{Me-}^{14}\text{C}$]methionine was determined as described by Salem *et al.* (1972). Results shown in parentheses were obtained by using extracts from the organism grown with added cyanocobalamin. In all cases, methionine formation was less than 3 nmol/h per mg of protein when homocysteine was omitted.

		Methionine formed (nmol/h per mg of protein) from			
		5- $^{14}\text{CH}_3\text{-H}_4\text{PteGlu}_1$		5- $^{14}\text{CH}_3\text{-H}_4\text{PteGlu}_3$	
		Aerobic	Anaerobic	Aerobic	Anaerobic
(a) <i>Cop. lagopus</i>	None	—	—	45	(49)
	<i>S</i> -Adenosylmethionine, (H) and methylcobalamin	—	1 (1)	—	(45)
	$\text{H}_4\text{PteGlu}_3$	—	(2)	—	—
	<i>S</i> -Adenosylhomocysteine	—	—	2	—
(b) <i>Cand. utilis</i>	None	(2)	5 (23)	84 (89)	92 (130)
	<i>S</i> -Adenosylmethionine, (H) and methylcobalamin	—	(23)	—	90 (131)
	$\text{H}_4\text{PteGlu}_3$	—	4	—	—
	<i>S</i> -Adenosylhomocysteine	—	—	1	—
(c) <i>B. megaterium</i>	None	—	—	5	56
	<i>S</i> -Adenosylmethionine, (H) and methylcobalamin	—	8	—	52
	$\text{H}_4\text{PteGlu}_3$	—	2	—	—
	<i>S</i> -Adenosylhomocysteine	—	—	—	1

methionine production during incubations under H_2 was increased by 40%. These effects were not intensified by supplementary amounts of *S*-adenosylmethionine, methylcobalamin and the reducing system (H).

Little methionine was produced from homocysteine and 5- $\text{CH}_3\text{-H}_4\text{PteGlu}_3$ with a *B. megaterium* extract, unless the incubation was done under H_2 (Table 2c), indicating the lack of an enzyme with the properties of *E. coli* cobalamin-independent transmethylase. Nevertheless, the enzyme in *B. megaterium* did not conform to the expected properties of a cobalamin-dependent transmethylase in its inactivity with 5- $\text{CH}_3\text{-H}_4\text{PteGlu}_1$ and in the failure of the cofactors *S*-adenosylmethionine, methylcobalamin and the reducing system (H) to increase methionine production from 5- $\text{CH}_3\text{-H}_4\text{PteGlu}_3$.

The inability of *S*-adenosylhomocysteine to substitute for homocysteine as a methyl acceptor from 5- $\text{CH}_3\text{-H}_4\text{PteGlu}_3$ is common to extracts of all three organisms. So also is the lack of stimulation

of methionine production by $\text{H}_4\text{PteGlu}_3$ when added to incubations containing 5- $\text{CH}_3\text{-H}_4\text{PteGlu}_1$ and homocysteine, suggesting the absence of an enzymic transfer of methyl groups from 5- $\text{CH}_3\text{-H}_4\text{PteGlu}_1$ to $\text{H}_4\text{PteGlu}_3$ in these organisms.

5- $\text{CH}_3\text{-H}_4\text{PteGlu}_3$ -homocysteine transmethylase in *B. megaterium*

During tests for the presence of this enzyme, it was observed that the specific activity increased with the amount of crude extract incubated with the substrates and Mg^{2+} only. Dialysis of the extract abolished transmethylase activity but supplementation of the dialysed preparation with *S*-adenosylmethionine (1 mM) partially restored specific activity, which was now independent of the concentration of extract used for the assay. About 50% of the maximum activity of the crude extract was lost during dialysis and was not restored by methylcobalamin or cofactors present in a heated extract of *B. megaterium*.

Crude ultrasonic preparations also lost about 50% of transmethyrase activity on storage at -20°C for 1 week.

Methylcobalamin-homocysteine transmethyrase

Extracts of all three organisms were tested for the ability to use methylcobalamin as a methyl donor to homocysteine as described by Salem *et al.* (1972). Such activity has been observed with purified preparations of the *E. coli* cobalamin-dependent homocysteine transmethyrase (Guest *et al.*, 1962; Weissbach *et al.*, 1964; Taylor, 1971); no cofactors were required.

With *B. megaterium* and *Cand. utilis* extracts, methionine formation from methylcobalamin and homocysteine was just detectable (4–6 nmol/h per mg of protein) above the non-enzymic reaction (13–20 nmol/h), but far less than was observed simultaneously with an extract of *E. coli* 3/62 containing an active vitamin B₁₂-dependent transmethyrase (42 nmol/h per mg of protein). No methylcobalamin-homocysteine transmethyrase activity was detected in *Cop. lagopus* extracts.

S-Adenosylmethionine as a methyl donor to homocysteine

S-Adenosylmethionine or *S*-methylmethionine have been suggested as final methyl donors to homocysteine by Shapiro and co-workers. Thus *S*-adenosylmethionine- or *S*-methylmethionine-homocysteine transmethyrase has been reported in extracts of *Aerobacter aerogenes* (Shapiro, 1956, 1962), *Candida albicans* (Balish & Shapiro, 1969; Mardon & Balish, 1971), *Saccharomyces cerevisiae* (Shapiro, 1958, 1965; Shapiro *et al.*, 1964; Botsford & Parks, 1969) and higher-plant seeds (Turner & Shapiro, 1961).

None of the extracts of the organisms in the present investigation catalysed the formation of significant amounts of [*Me*-¹⁴C]methionine when incubated with *S*-adenosyl[*Me*-¹⁴C]methionine and homocysteine. Further, the net synthesis of methionine would require the re-methylation of *S*-adenosyl-

homocysteine without, of course, the intermediate formation of methionine itself. However, attempts to re-methylate *S*-adenosylhomocysteine by using cell-free extracts *in vitro* have been unsuccessful (Duerre & Schlenk, 1962) and, in the present study, no methylation of *S*-adenosylhomocysteine by 5-CH₃-H₄PteGlu₃ has been detected.

Nature of endogenous folates

In each of the organisms in the present investigation, methionine is synthesized by transmethylation to homocysteine from a polyglutamate folate. In the generation of the methyl group, 5,10-methylenetetrahydrofolate reductase is active with either a conjugated folate or the monoglutamate form (Table 1) and the same is true for serine-tetrahydrofolate 5,10-hydroxymethyltransferase (serine transhydroxymethylase, EC 2.1.2.1; Table 3). A pathway can therefore be traced from serine to methionine in which the methyl group is generated on and transferred directly from a polyglutamate folate coenzyme. Such folates should then be expected to occur endogenously in each organism. Table 4 shows the results of analysis by t.l.c. and bioautography of microbiologically active material prepared from cell-free extracts by column chromatography on TEAE-cellulose as described by Salem *et al.* (1972).

Only polyglutamate forms were found, as indicated by their changed chromatographic behaviour, after treatment with a chicken pancreas conjugase preparation. In each organism, one folate (II, V and VIII), stable to air, is chromatographically identical with 5-HCO-H₄PteGlu₃. Conjugase treatment of folates II, V and VIII gave a final product chromatographically similar to 5-HCO-H₄PteGlu₁. The same product resulted from conjugase treatment of components I (from *B. megaterium*) and IV (from *Cand. utilis*); these folates, before enzymic hydrolysis, have the chromatographic properties of 5-HCO-H₄PteGlu₂. A third microbiologically active component (VI) in *Cand. utilis* is probably unreduced, unsubstituted PteGlu₃, and on incubation with conjugase gave a final product that co-chromatographed

Table 3. *Serine transhydroxymethylase in microbial extracts*

Extracts of organisms grown in minimal medium were incubated with L-[3-¹⁴C]serine and were assayed for serine transhydroxymethylase by the method of Taylor & Weissbach (1965).

Extract	Folate present	...	5,10-Methylenetetrahydrofolate formed (nmol/h per mg of protein)	
			H ₄ PteGlu ₁	H ₄ PteGlu ₃
<i>B. megaterium</i>			2310	2060
<i>Cand. utilis</i>			740	790
<i>Cop. lagopus</i>			152	146

Table 4. *Chromatographic analysis of endogenous folates*

The overall method of analysis was as described by Salem *et al.* (1972). The supernatant fluids after centrifugation of the cell-free extracts (25000 g; 15 min) were filtered through Sephadex G-25 by using 20 mM-potassium phosphate buffer, pH 7.8, containing 15 mM-2-mercaptoethanol, to separate low-molecular-weight material. Fractions with growth activity for *Lactobacillus casei* that were eluted behind the peak of excluded material were taken for the separation of folates by column chromatography on TEAE-cellulose. Fractions were monitored for the presence and nature of the folates by t.l.c. and bioautography with *L. casei*. Solvent A: 5% (w/v) citric acid adjusted to pH 9.0 with NH₃ solution and saturated with 3-methylbutan-1-ol at 25°C; solvent B: formic acid-ethanol-water (1:15:35, by vol.).

		<i>R_F</i> value							
Organism	...	<i>B. megaterium</i>			<i>Cand. utilis</i>			<i>Cop. lagopus</i>	
Folate fraction	...	I	II	III	IV	V	VI	VII	VIII
T.l.c. before conjugase treatment:									
Solvent A		0.78	0.92	0.41	0.80	0.91	0.55	0.73	0.91
Solvent B		0.82	0.84	0.66	—	—	—	0.68	0.83
T.l.c. after conjugase treatment:									
Solvent A		0.67	0.68	0.30	0.69	0.69	0.23	0.41	0.69
Solvent B		0.73	0.76	0.50	—	—	—	0.57	0.76

		<i>R_F</i> value	
Synthetic folate	Solvent ...	A	B
5-HCO-H ₄ PteGlu ₃		0.91	0.84
5-HCO-H ₄ PteGlu ₂		0.79	—
5-HCO-H ₄ PteGlu ₁		0.68	0.76
PteGlu ₃		0.55	—
PteGlu ₁		0.22	—

with PteGlu₁. Other conjugated forms in *B. megaterium* (III) and *Cop. lagopus* (VII), which were active for the growth of *Lactobacillus casei*, have not been identified. Since the detector organism, *L. casei*, used in the separation of naturally occurring folates does not respond to folates with more than three glutamate residues (Blakley, 1969), higher conjugates would not be detected here. Microbiological assay of crude extracts before and after conjugase treatment indicated that up to 50% of endogenous folates in these organisms comprise conjugates higher than triglutamates.

Relationship between cobalamin and the biosynthesis of methionine

The results of the endogenous folate analyses are consistent with the requirement by each organism for a polyglutamate form in methionine synthesis. The enzymic tests suggest the operation of a cobal-

amin-independent pathway to methionine in *Cop. lagopus* and *Cand. utilis* grown in minimal medium, in a way similar to the sequence in *E. coli* grown without added vitamin B₁₂ (Woods *et al.*, 1965). Although growth of *Cand. utilis* with cobalamin produces a curious alteration in the specificity of 5-methyltetrahydrofolate-homocysteine transmethy-lase, which is now able to use 5-CH₃-H₄PteGlu₁, this effect is much less pronounced than with *E. coli* (Guest *et al.*, 1964b) and does not confer the ability to use H₄PteGlu₁ as a coenzyme in the overall C₁-transfer from serine to homocysteine. The increased sensitivity to air of the transmethy-lase in *Cand. utilis* grown with added vitamin B₁₂ (Table 2b) is also less marked than in *E. coli*, where aerobic incubation of extracts of the cobalamin-grown organism gives only 1% of the vitamin B₁₂-dependent transmethy-lase activity found under anaerobic conditions (Dawes & Foster, 1971). Further, the activation of a vitamin B₁₂-dependent homocysteine trans-methy-lase, similar to that in *E. coli*, by growth of

Table 5. *Vitamin B₁₂ and the methylation of homocysteine in some organisms*

References: a, Guest *et al.* (1964b); b, Taylor & Weissbach (1969b); c, Whitfield *et al.* (1970); d, Salem *et al.* (1972); e, Cauthen *et al.* (1966); f, Morningstar & Kisliuk (1965); g, Cauthen *et al.* (1967); h, Lago & Demain (1969a,b); i, Ohmori *et al.* (1971); j, Burton & Sakami (1967); k, Burton *et al.* (1969); l, Selhub *et al.* (1969); m, Burton & Sakami (1969); n, Dodd & Cossins (1970); o, Mangum *et al.* (1969); p, Burke *et al.* (1971).

Organism	Endogenous cobalamin formation	Methylation of homocysteine		References
		Cobalamin-independent	Cobalamin-dependent	
<i>E. coli</i>	—	+	+	a, b, c
<i>B. subtilis</i>	—	+	—	d
<i>B. megaterium</i>	+	—	+	e
<i>Salmonella typhimurium</i>	±	+	+	f
<i>Aerobacter aerogenes</i>	+	+	+	g
<i>Rhodopseudomonas spheroides</i>	+	—	+	h
<i>Pseudomonas denitrificans</i>	+	—	+	i
<i>Streptomyces olivaceus</i>	+	—	+	j, k
<i>Saccharomyces cerevisiae</i>	—	+	—	
<i>Cand. utilis</i>	—	+	—	
<i>Cop. lagopus</i>	—	+	—	
<i>Neurospora crassa</i>	—	+	—	k, l
Green plants	—	+	—	m, n
Mammals	—	—	+	o, p

* Indicates homocysteine transmethylase specific for a conjugated folate.

Cand. utilis with added vitamin B₁₂ is not supported by tests for the use of methylcobalamin as methyl donor to homocysteine.

The requirement for anaerobic conditions for methionine production from 5-CH₃-H₄PteGlu₃ and homocysteine by *B. megaterium* extract and the requirement for S-adenosylmethionine by the dialysed extract for this transmethylation strongly suggests the operation of a vitamin B₁₂-dependent transmethylase. This was supported by the results of growth tests, in which methionine partially overcame the growth-inhibitory effect of the anilide analogue of vitamin B₁₂. In the absence of the analogue, *B. megaterium* grew to a final density of 0.31 mg dry wt./ml, whereas with added vitamin B₁₂-anilide growth to only 0.08 mg dry wt./ml occurred. L-Methionine (1 mM) increased the final density of a culture containing the inhibitor to 0.13 mg dry wt./ml. However, the homocysteine transmethylase in *B. megaterium* differs from the cobamide enzymes of *E. coli*, *Salmonella typhimurium* and mammalian liver in its comparative inactivity with 5-CH₃-H₄PteGlu₁ as substrate (Guest *et al.*, 1964b; Cauthen *et al.*, 1966; Burke *et al.*, 1971). A further difference from the *E. coli* transmethylase is the relatively low capacity of the *B. megaterium* extract for methionine synthesis from methylcobalamin and homocysteine.

As methylcobalamin is a postulated intermediate in the vitamin B₁₂-dependent methylation of homocysteine (Taylor & Weissbach, 1969a; Burke *et al.*, 1970), evidence for such a mechanism was sought by extracting cobalamin from the three organisms by the method of Salem *et al.* (1972), and comparing the results with those of the same procedures when applied to *E. coli* strain B/r. *Cand. utilis*, *Cop. lagopus* and *E. coli* were grown with added cyanocobalamin for this experiment.

Bioautography of the isolated cobalamins after co-chromatography with authentic compounds (Salem *et al.*, 1972) revealed methylcobalamin as the major component in *E. coli*, together with traces of hydroxo-cobalamin. *B. megaterium*, *Cand. utilis* and *Cop. lagopus* contained hydroxo-cobalamin and 5'-deoxyadenosylcobalamin in addition to unchanged cyanocobalamin, but no methylcobalamin. These results are further indications of dissimilarities between the mechanisms of homocysteine methylation in these three organisms and the vitamin B₁₂-dependent transmethylation in *E. coli*. Nevertheless, the appearance of other cobalamins in extracts of *Cand. utilis* and *Cop. lagopus* after growth with cyanocobalamin suggests that the three organisms in this investigation can metabolize vitamin B₁₂, although the functions of the products are unknown.

Comparison with other organisms

The present results, together with recent surveys of methionine synthesis in other organisms (Table 5), demonstrate the differences in mechanism that occur between classes and even within the same class of organism. Nevertheless, in those organisms that produce the vitamin, a cobalamin-dependent methylation of homocysteine has been generally found, and some properties of the transmethylese from *B. megaterium* appear to support this trend. Conversely, with the striking exceptions of *E. coli* and mammals, cobalamin-less organisms do not possess a vitamin B₁₂-dependent transmethylese, a pattern extended by results with *Cand. utilis* and *Cop. lagopus*.

The transmethylese from *B. megaterium*, in its requirements for *S*-adenosylmethionine and anaerobic conditions, appears to be the first instance of a cobalamin-dependent transmethylese with strict specificity for a polyglutamate folate. The general question of folate specificity requires consideration not only of the efficiency of a particular enzymic reaction in terms of thermodynamic and kinetic parameters, but also of the relative availability within the organism of polyglutamate folates and their unconjugated forms. In the latter respect, the occurrence of only polyglutamate folates in *B. megaterium*, *Cand. utilis* and *Cop. lagopus* under conditions of active methionine synthesis is consistent with the observed specificity of the respective homocysteine transmethylese for a 5-methyltetrahydropteroyl-polyglutamate. A similar relationship occurs in germinating pea seedlings, where the high affinity of the homocysteine transmethylese for 5-CH₃-H₄PteGlu₁ (Dodd & Cossins, 1970) agrees with the finding that this derivative is the major form of folate in this organism (Roos *et al.*, 1968).

Although the methylation of homocysteine by *S*-adenosylmethionine (or *S*-methylmethionine) has been reported in a number of micro-organisms (Shapiro *et al.*, 1963) as a major route to methionine, no significant activity has been detected in the organisms investigated in the present work. The position of *S*-adenosylmethionine-homocysteine transmethylese in methionine metabolism remains unclear, as the re-methylation of *S*-adenosylhomocysteine required for net methionine synthesis cannot be demonstrated. To date, the only known route to *S*-adenosylmethionine is via methionine and ATP (Cantoni, 1953).

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