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Folic Acid and the Methylation of Homocysteine by *Bacillus subtilis*

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1. Cell-free extracts of *Bacillus subtilis* synthesize methionine from serine and homocysteine without added folate. The endogenous folate may be replaced by tetrahydropteroyltriglutamate or an extract of heated *Escherichia coli* for the overall C_1 transfer, but tetrahydropteroylmonoglutamate is relatively inactive. 2. Extracts of *B. subtilis* contain serine transhydroxymethylase and 5,10-methylenetetrahydrofolate reductase, which are non-specific with respect to the glutamate content of the folate substrates. Methyl transfer to homocysteine requires a polyglutamate folate as methyl donor. These properties are not affected by growth of the organism with added vitamin B_{12}. 3. The synthesis of methionine from 5-methyltetrahydropteroyltriglutamate and homocysteine has the characteristics of the cobalamin-independent reaction of *E. coli*. No evidence for a cobalamin-dependent transmethylation was obtained. 4. *S*-Adenosylmethionine was not a significant precursor of the methyl group of methionine with cell-free extracts, neither was *S*-adenosylmethionine generated by methylation of *S*-adenosylhomocysteine by 5-methyltetrahydrofolate. 5. A procedure for the isolation and analysis of folic acid derivatives from natural sources is described. 6. The folates isolated from lysozyme extracts of *B. subtilis* are sensitive to folic acid conjugase. One has been identified as 5-formyltetrahydropteroyltriglutamate; the other is possibly a diglutamate folate. 7. A sequence is proposed for methionine biosynthesis in *B. subtilis* in which methyl groups are generated from serine and transferred to homocysteine by means of a cobalamin-independent pathway mediated by conjugated folate coenzymes.

The methyl group of methionine has been shown to arise in various organisms by the enzymic reduction of 5,10-methylenetetrahydrofolate, the C_1 unit being derived from C-3 of serine via serine transhydroxymethylase (L-serine–tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2.1.2.1) (Larrabee & Buchanan, 1961; Cauthen *et al.*, 1966, 1967; Sakami & Ukstins, 1961; Kutzbach & Stokstad, 1966, 1967). In *Escherichia coli*, the enzymic generation of methyl groups can occur with either $H_4PteGlu_1$† or $H_4PteGlu_3$ as C_1-unit carrier (Guest *et al.*, 1964*a*).

E. coli has alternative enzymic mechanisms for the methylation of homocysteine by 5-methyltetrahydrofolate (Woods *et al.*, 1965). One of these involves a cobamide-containing enzyme (Guest *et al.*, 1964*b*) which requires *S*-adenosylmethionine and $FADH_2$ as cofactors (Rosenthal & Buchanan, 1963; Foster *et al.*, 1964*a*). The other enzyme does not contain cobalamin (Foster *et al.*, 1964*b*) and requires only Mg^{2+} ions (Guest *et al.*, 1964*b*) and P_i (Whitfield *et al.*, 1970). Transmethylation by the latter enzyme specifically requires a polyglutamate folate, e.g. 5-CH_3-$H_4PteGlu_3$ (Foster *et al.*, 1964*b*), whereas the cobamide enzyme is also active with 5-CH_3-$H_4PteGlu_1$. Since the organism is unable to synthesize significant quantities of vitamin B_{12} (Foster *et al.*, 1964*b*; Woods *et al.*, 1965), the cobamide enzyme operates only when vitamin B_{12} is supplied exogenously. However, the apoenzyme, present throughout growth on minimal medium, may be activated by the addition of cobalamin to either the growth medium or ultrasonic extracts (Kisliuk & Woods, 1960; Guest & Woods, 1962; Takeyama *et al.*, 1961).

Similar dual pathways have been proposed for methyl transfer from 5-methyltetrahydrofolate to homocysteine in *Salmonella typhimurium* (Cauthen *et al.*, 1966) and *Aerobacter aerogenes* (Morningstar & Kisliuk, 1965). However, these organisms do contain endogenous cobamide.

It is worthwhile to discover whether, like *E. coli*, other bacteria which do not synthesize cobalamin or

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† Abbreviations: for members of the folic acid group, based on $PteGlu_1$ for pteroylglutamate: $H_4PteGlu_1$, tetrahydropteroylmonoglutamate; $H_4PteGlu_3$, tetrahydropteroyltriglutamate, etc.; derivatives of these compounds are indicated as, e.g. 5-CH_3-$H_4PteGlu_3$ (the term folate is used in this paper in reference to both simple and conjugated members of the folic acid family of vitamins); preparation EHC, a soluble extract of a heated suspension of *E. coli*.

require added vitamin B_{12} for growth possess a vitamin B_{12}-dependent homocysteine transmethylase in addition to a functional transmethylase independent of cobalamin. The present paper concerns the mechanism of methylation of homocysteine by *Bacillus subtilis*, an organism found to contain insignificant quantities of cobalamin when grown in the absence of the vitamin.

Materials and Methods

Organisms

Stock cultures of *B. subtilis* (N.C.I.B. 3610) and strains of *E. coli* were maintained on slopes of nutrient agar (Oxoid CM3), sub-cultured monthly, incubated for 18h at 37°C and stored at 4°C. *E. coli* strains 121/176 and 113/3 are cobalamin or methionine auxotrophs (Davis & Mingioli, 1950) and lack 5-CH_3-H_4PteGlu$_3$–homocysteine transmethylase (Foster *et al.*, 1964*b*). *E. coli* strain 3/62, which lacks 5,10-methylenetetrahydrofolate reductase (Guest *et al.*, 1964*a*), responds to methionine but not to cobalamin or homocysteine.

Media and growth

B. subtilis was grown in liquid culture in a minimal-salts medium (Davis & Mingioli, 1950) containing glucose (40mM) and iron citrate (1%, w/v). Erlenmeyer flasks (1000ml) containing 200ml of growth medium were shaken at 37°C after inoculation with about 6×10^8 bacteria from an 18h culture in the same medium. For medium supplemented with cobalamin, the vitamin was added as a sterile solution to the autoclaved medium. Growth was assessed with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex, U.K.).

E. coli strains 3/62 and 121/176 were grown on solid defined media in enamelled trays as described by Guest *et al.* (1960). The basal medium was supplemented with DL-methionine (0.5mM). The medium for growth of *E. coli* 3/62 was further supplemented with cyanocobalamin (15nmol/500ml of medium).

Cell-free extracts

Ultrasonic extracts were prepared and stored as described by Cauthen *et al.* (1966). The protein content was determined spectrophotometrically by the method of Layne (1957).

Lysozyme lysates of *B. subtilis* were prepared by using a freeze–thaw method based on that of Ron *et al.* (1966). A thick suspension of bacteria (25mg dry wt./ml) in 0.15M-NaCl containing 2-mercaptoethanol (15mM) and egg-white lysozyme (15μg/ml) was repeatedly frozen in liquid N_2 and thawed slowly in cold running water until the suspension was almost completely cleared. Three freeze–thaw cycles were normally sufficient. The supernatant fluid after centrifuging (25000*g*; 20min) at 0°C was retained for the isolation of folates.

An extract of heated *E. coli* (preparation EHC), containing natural forms of folic acid, was prepared from acetone-dried organisms of strain PA15 (grown in the absence of added cobalamin) as described by Guest *et al.* (1960). It is interchangeable with H_4PteGlu$_3$ as a cofactor in methionine synthesis by enzymic extracts of *E. coli*.

Isolation and chromatography of natural cobalamins

A modification of the procedure of Barker *et al.* (1960) was used for concentrating cobamide coenzymes. All operations were performed in dim red light at room temperature. A thick paste of *B. subtilis* was heated with ethanol (4vol.) for 20min at 80°C. After centrifuging, the supernatant liquid was evaporated under reduced pressure to a small volume and washed twice with ether (1vol.). The aqueous layer was extracted twice with aq. 85% (w/v) phenol (1vol.); the washed phenol layer was then shaken with water (0.2vol.), ether (3vol.) and acetone (1vol.). After removal of the heavy aqueous layer, the remainder was extracted twice more with water (0.2vol.). After washing three times with ether (1vol.) to remove phenol, the combined aqueous layers were adjusted to pH9 with ammonia solution and passed through a column (5cm$\times$0.5cm^2) of Dowex 1 resin (X8; 100–200 mesh; OH^- form) to remove methionine. The receiver contained solid CO_2 for neutralization of the alkali. The eluate was evaporated under reduced pressure and chromatographed on Whatman no. 1 paper in descending fashion, with, as solvent, butan-2-ol–acetic acid–water (100:3:50, by vol.). The positions of areas containing microbiologically active cobalamins were detected bioautographically by applying the dried paper to the surface of minimal medium containing agar (2%, w/v), seeded with *E. coli* 113/3 (20mg dry wt./l). Triphenyltetrazolium chloride (0.1%, w/v) was used to facilitate detection of the growth zones of the test organism. Synthetic cobalamins (200pg) were applied as reference compounds. As little as 10pg of a cobalamin was detected in this way.

Tests for methionine synthesis

The conditions used for testing methionine synthesis were based on previous work with *E. coli* in this laboratory (Foster *et al.*, 1964*b*). When serine was the ultimate source of C_1 units, the basal reaction mixture, M1, contained, in a final volume of 1ml: potassium phosphate buffer (pH7.8), 100mM; DL-homocysteine, 6.7mM; L-serine, 5mM; ATP, 5mM; glucose, 20mM; $MgSO_4$, 5mM; pyridoxal phosphate,

0.5mB; a reducing system (H) containing FAD, 75nmol, NAD, 250nmol, ethanol, 50μmol and yeast alcohol dehydrogenase, 90μg. Enzymic extracts (1–3mg of protein) were added. Endogenous folate coenzymes were removed by first passing the extracts through columns of Dowex 1 (X8; 100–200 mesh; Cl^- form), allowing at least 4cm^3 of packed resin/100mg of protein. The source of folic acid cofactor was either preparation EHC (equivalent to 14mg dry wt. of *E. coli*) or $H_4PteGlu_1$ (460μM) or $H_4PteGlu_3$ (72μM).

The complete reaction mixtures were incubated at 37°C for 1h in an atmosphere of H_2 and the enzymic reaction was terminated by heating for 3min at 100°C. The supernatant fluids, after centrifuging, were used for the microbiological assay of L-methionine with *Leuconostoc mesenteroides* P60 (Gibson & Woods, 1960). In each test, a value (5–10nmol) representing the amount of L-methionine found to be present initially in the complete incubation mixtures was subtracted from each recorded experimental value.

When 5-[^{14}C]methyltetrahydrofolate was the source of C_1 units, the basal incubation mixture, M2, contained, in a final volume of 0.1ml: potassium phosphate buffer (pH7.8), 100mM; DL-homocysteine, 22mM; 2-mercaptoethanol, 21mM; $MgSO_4$, 4.5mM. Enzymic extract containing 0.5–2.0mg of protein was added. Substrates were either (±)-5-$^{14}CH_3$-$H_4PteGlu_1$ (0.5μCi/μmol), 1.1mM, or (±)-5-$^{14}CH_3$-$H_4PteGlu_3$ (0.5μCi/μmol), 0.3–3.0mM. Other additions, where indicated, were: *S*-adenosylmethionine, 20μM; the reducing system (H) described above; methylcobalamin, 10μM; $H_4PteGlu_3$ or $H_4PteGlu_1$, 6mM.

The reaction mixtures were incubated at 37°C in an atmosphere of H_2 for between 30 and 120min. After addition of 0.4ml of water at 0°C to each, the incubated solutions were passed through columns of Dowex 1 resin (X8; 100–200 mesh; Cl^- form) to remove unchanged folate, as described by Foster *et al.* (1964*a*). After the columns had been washed twice with 1ml of water, samples (0.2ml) of the combined effluent and washings were dissolved in 5ml of scintillation fluid containing 0.7% (w/v) 5-(4-biphenylyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole and 8% (w/v) naphthalene in a solvent of toluene and 2-methoxyethanol (3:2, v/v). Radioactivity was measured at 90% efficiency with a Beckman model LS200 liquid-scintillation spectrometer. None of the materials present caused significant quenching of [*Me*-^{14}C]-methionine. Methionine was the only radioactive product detected radioautographically after descending chromatography on Whatman no. 1 paper with, as solvent, butan-1-ol–acetic acid–water (1:1:2, by vol.). The amount of L-methionine formed was calculated from the initial specific radioactivity of the 5-methyltetrahydrofolate and agreed with values obtained by occasional random microbiological assays.

For measurement of methyl transfer to homocysteine from methylcobalamin, the basal reaction mixture, M3, contained, in a final volume of 0.25ml: potassium phosphate buffer (pH7.5), 85mM; DL-homocysteine, 8mM; 2-mercaptoethanol, 24mM; [^{14}C]methylcobalamin (2.0μCi/μmol), 1.0mM. Enzymic extract, containing 1–3mg of protein, was added and the mixture incubated in the dark for 2h at 37°C under H_2. The enzymic reaction was terminated by addition of 1M-KOH (0.05ml) and the mixtures were passed through columns of Dowex 1 resin (X8; 100–200 mesh; OH^- form). Unchanged methylcobalamin was washed through with 0.1M-KOH (4×0.75ml) and the columns were then eluted with 2M-HCl (2×0.5ml and 2×0.75ml) to release adsorbed methionine. Samples (1ml) of the combined acid effluent were evaporated to dryness, the residue was redissolved in water (0.2ml) and 5ml of scintillation fluid added for measurement of radioactivity. All manipulations with methylcobalamin were performed in red light. The amount of methionine formed was calculated from the initial specific radioactivity of the methylcobalamin.

Test for the degradation of S-adenosylmethionine

The use of *S*-adenosylmethionine as a methyl donor was followed by assessing the conversion of *S*-adenosyl[*Me*-^{14}C]methionine into less cationic products. The basal reaction mixture, M4, contained, in a final volume of 0.1ml: potassium phosphate buffer (pH7.8), 100mM; *S*-adenosyl[*Me*-^{14}C]methionine (20μCi/μmol), 0.5–5mM; the reducing system (H) described above. Either DL-homocysteine (20mM) or (±)tetrahydrofolate (1–6mM) was added as methyl acceptor. Enzymic extract was added to a final protein concentration of 3–10mg/ml and the mixture incubated at 37°C in an atmosphere of H_2 for 1h. After incubation, 0.4ml of water at 0°C was added to each tube and the contents were passed through columns of Dowex 50 (X8; 200–400 mesh; Li^+ form) to remove unchanged *S*-adenosylmethionine. The columns were washed twice with 1ml of water and the radioactivity of samples (0.2ml) of the eluate was measured as described above.

Determination of folic acid

Microbiological assays with *Lactobacillus casei* (N.C.I.B. 6375), *Streptococcus faecalis* R (N.C.I.B. 6459) and *Pediococcus pentosaceus* (N.C.I.B. 7837) were employed for the determination of various forms of folic acid. *Lb. casei* grows with mono-, di- and triglutamate forms of folate but not with pteroate; *S. faecalis* responds to pteroate, pteroylglutamate and their derivatives but not to the di- and tri-glutamate; *P. pentosaceus* requires a reduced form of folate and

does not respond to any unreduced compound. None of the organisms responds to pteroylheptaglutamate.

A single basal medium (Metz *et al.*, 1968) was used for assays with all three organisms and for bioautographic work. Assay tubes contained, in a final volume of 5ml: the sample for assay together with water to 2.4ml, and double-strength basal medium (2.5ml) containing ascorbic acid (20mM) as antioxidant. Standards containing $PteGlu_1$ or leucovorin (synthetic 5-HCO-$H_4PteGlu_1$), 0.04–1.0nM, were included in each assay. Leucovorin is a mixture of two stereoisomers, only one of which supports growth of the assay organisms (Cosulich *et al.*, 1952). The tubes were autoclaved for 5min at 121°C and inoculated with about 10^6 cells from a diluted suspension of the washed organism grown overnight in nutrient broth. After incubation upright in air for 40–42h at 37°C, the growth was assessed with an EEL photoelectric colorimeter. The uninoculated medium was used as a blank.

Polyglutamates of the folic acid group were hydrolysed to lower conjugates by treatment with a conjugase preparation from dried chicken pancreas (Difco Laboratories, Detroit, Mich., U.S.A.), the protein fraction which precipitates between 40% and 80% saturation of ammonium sulphate at pH7 being used. This preparation contained 20mg of protein/ml and converted 9.6nmol of $PteGlu_3$/3h per mg of protein into a form active for the growth of *S. faecalis*. The reaction mixture for the hydrolysis of isolated folates contained, in a final volume of 1ml: tris–HCl buffer (pH7.9), 0.1M; $CaCl_2$, 10mM; 2-mercaptoethanol, 20mM; the isolated folate, 0.5–1nmol; the conjugase preparation, 5mg of protein. Tubes were incubated upright in air at 37°C for 24h. After termination of the reaction by heating (100°C; 3min) and removal of the conjugase protein by centrifugation, samples (5μl) of the supernatant were taken for t.l.c. and bioautography with *Lb. casei*.

Thin-layer chromatography of folates

Routine chromatography of folates was performed with layers of cellulose (MN300; Macherey, Nagel and Co., Duren, Germany) at an average thickness of 250μm. Three solvent systems were used in this work: A, 5% (w/v) citric acid adjusted to pH9.0 with NH_3 (sp.gr. 0.88) and saturated with 3-methylbutan-1-ol at 25°C; B, formic acid–ethanol–water (2:30:70, by vol.); C, ethyl acetate–2-methylpropan-2-ol–monoethanolamine–water (2:3:1:3, by vol.). Each solvent contained 2-mercaptoethanol at a final concentration of 40mM. Samples (5–10μl) containing 1–5pmol of folate were applied and dried in a stream of air (or, for isolated folates and labile derivatives, O_2-free N_2). Synthetic folates were used as external markers. The solvent front was allowed to proceed to a line drawn 10cm from the origin.

For anion-exchange t.l.c. on Whatman DE41 cellulose, the concentration-gradient technique of Rybicka (1962) was followed.

The positions of folates after chromatography were detected bioautographically. After removal of the solvent from the chromatograms by a stream of cold air, the plate was placed, surface layer upwards, in a Perspex tray (21cm×21cm) and covered with molten agar medium at 45°C, containing: double-strength folic acid assay medium, 90ml; agar (Oxoid no. 3), 4g; triphenyltetrazolium chloride (1%, w/v), 1.8ml; water to 180ml; and inoculated with *Lb. casei*. The inoculum was 1ml of a washed suspension (approx. 10^8 cells/ml) in 0.15M-NaCl of *Lb. casei* cultured overnight in nutrient broth. The bioautograms were developed at 37°C for 15–20h, when red growth zones marked areas of the plate carrying microbiologically active folates. Background colour was not significant provided the depth of agar was less than 3mm above the glass plate.

Chemicals

(±)-5-[^{14}C]Methyltetrahydrofolates were prepared by reduction of (±)-5,10-[^{14}C]methylenetetrahydrofolates with $NaBH_4$ and purified by column chromatography as described by Guest *et al.* (1964*a*). Solutions in potassium phosphate buffer, pH7.5 (50mM), containing 2-mercaptoethanol (7mM), were standardized spectrophotometrically (by using ϵ = 28000 litre·mol^{-1}·cm^{-1} at 290nm) and the specific radioactivity was determined by scintillation counting of diluted samples.

Pteroyltriglutamic acid was a gift from Professor E. L. R. Stokstad (Department of Nutritional Sciences, University of California, Berkeley, U.S.A.). For some experiments, it was purified by chromatography on a column of cellulose powder with 0.1M-sodium phosphate buffer (pH7.0), saturated with 3-methylbutan-1-ol, as solvent. The purified material was precipitated several times from neutral solution with acetic acid and finally freeze-dried.

Tetrahydrofolates, 10-formylfolates and 10-formyltetrahydrofolates were prepared from $PteGlu_1$ and $PteGlu_3$ as described by Jones *et al.* (1961). 5-Formyltetrahydrofolates were prepared from the corresponding 10-formyltetrahydrofolates by addition of 2-mercaptoethanol to 0.4M, adjustment to pH8.2 and autoclaving for 1h at 121°C. After cooling and neutralization, the compounds were purified by chromatography on a column of TEAE-cellulose (OH^- form) with a gradient from water to 1M-ammonium acetate, pH6.1. Fractions containing 5-formyltetrahydrofolate were identified by the increase in E_{350} when a sample (0.5ml) was adjusted to pH1.0 with conc. HCl (Rabinowitz, 1960).

Cyanocobalamin was crystalline material obtained from British Drug Houses Ltd., Poole, Dorset, U.K.

Methylcobalamin was a gift from Dr. E. L. Smith, and 5'-deoxyadenosylcobalamin was supplied by Dr. L. Mervyn (Glaxo Laboratories, Greenford, Middlesex, U.K.). ATP, NAD and FAD were products of Sigma Chemical Corp., St. Louis, Mo., U.S.A. Solutions of DL-homocysteine (Fluka AG Chemische Fabrik, Buchs SG, Switzerland) were prepared immediately before use.

Results

Cobamide content of B. subtilis

The organism was taken from exponential growth in a minimal medium containing only mineral salts and glucose. Cobalamins were released either by heating a thick suspension of bacteria at pH 6.5 in the presence of 10mM-KCN and subsequent papain digestion (Foster *et al.*, 1964*b*), or by acid extraction at 100°C in 0.33M-HCl and 1% KCN (Volcani *et al.*, 1961). The supernatant fluids after centrifuging were assayed for cyanocobalamin with *Euglena gracilis* var. *bacillaris* (Foster *et al.*, 1964*b*) and with *E. coli* 113/3 (Foster *et al.*, 1961). *B. subtilis* contains only traces of endogenous vitamin B_{12} (0.1–11 ng/g dry wt. of organism). Paper chromatography and bioautography showed that ethanol extracts of *B. subtilis* after growth with exogenous cyanocobalamin (5 μg/l) contain hydroxocobalamin, 5'-deoxyadenosylcobalamin and unchanged cyanocobalamin but no methylcobalamin. Growth was unaffected by the addition of cyanocobalamin (2.5 μg/l) to the minimal medium.

Overall conversion of serine and homocysteine into methionine by ultrasonic extracts

Ultrasonic extracts of *B. subtilis* grown in the absence of exogenous vitamin B_{12} catalysed the transfer of C_1 units from serine to homocysteine at a rate of 60 nmol/h per 3 mg of protein. An added folate coenzyme was not required. However, after removal of endogenous folates by passage of the extracts through a column of Dowex 1, methionine formation depended on the addition of $H_4PteGlu_3$ or preparation EHC; $H_4PteGlu_1$ was inactive as C_1-group carrier (Table 1). Growth of the organism with added vitamin B_{12} (2.5 μg/l) did not significantly influence the activity of Dowex-treated extracts with these folates. The addition of methionine (2mM) to the growth medium, however, resulted in a decrease by 50% of overall C_1-group transfer by extracts with $H_4PteGlu_3$.

Serine–tetrahydrofolate 5,10-hydroxymethyltransferase (serine transhydroxymethylase)

Serine transhydroxymethylase was measured radioisotopically in crude extracts by the method of Taylor & Weissbach (1965). Conditions were as employed for the assessment of overall C_1-group transfer from serine to homocysteine with respect to protein concentration, pH and serine concentration. These conditions, although not necessarily optimum for the enzyme, gave activities more directly and quantitatively comparable with estimates of methionine synthesis by extracts. The activity of serine transhydroxymethylase in ultrasonic extracts of *B. subtilis* was 2410 nmol/h per mg of protein with $H_4PteGlu_1$ and 2030 nmol/h per mg of protein with $H_4PteGlu_3$ as C_1-group acceptor. These values were not significantly affected by growth of the organism with added vitamin B_{12}.

5-Methyltetrahydrofolate–FAD oxidoreductase (5,10-methylenetetrahydrofolate reductase, EC 1.1.1.68)

Extracts of the methionine auxotroph, *E. coli* 3/62, specifically lack this reductase (Guest *et al.*, 1964*a*). Ultrasonic extracts of *B. subtilis* supported the synthesis of methionine from serine and homocysteine by a cell-free preparation of *E. coli* 3/62 (grown in the presence of vitamin B_{12}) with either

Table 1. *5,10-Methylenetetrahydrofolate reductase and 5-methyltetrahydrofolate–homocysteine transmethylase in extracts of B. subtilis*

Ultrasonic extracts, each containing 3 mg of protein, were incubated in mixture M1 containing serine and homocysteine. Incubations were for 1 h at 37°C under H_2. All extracts were first passed through columns of Dowex 1 (Cl^- form) to remove endogenous folates.

Extracts	L-Methionine formed (nmol) by using as folate coenzyme: None	$H_4PteGlu_1$	$H_4PteGlu_3$	Preparation EHC
*B. subtilis**	5	4	54	42
E. coli 3/62	3	9	5	16
E. coli 121/176	4	6	17	18
B. subtilis+*E. coli* 3/62	11	109	102	91
B. subtilis+*E. coli* 121/176	14	36	296	84

* The crude extract, before treatment with Dowex 1 resin, produced 60 nmol of L-methionine/h per 3 mg of protein in this assay in the absence of added folate coenzyme or ancillary extract.

$H_4PteGlu_1$ or $H_4PteGlu_3$ as folate coenzyme (Table 1). Since the *E. coli* 3/62 extract can form methionine from 5-CH_3-$H_4PteGlu_1$ or 5-CH_3-$H_4PteGlu_3$ but cannot produce these derivatives from tetrahydrofolates, there is 5,10-methylenetetrahydrofolate reductase activity in *B. subtilis* extracts. Moreover, this showed no specificity towards conjugated folates. These results were unaltered by growth of *B. subtilis* with added vitamin B_{12}.

5-Methyltetrahydropteroyltriglutamate–homocysteine transmethylase (EC 2.1.1.x)

Extracts of *E. coli* 121/176 grown in minimal medium supplemented with methionine only are deficient in the cobalamin-independent homocysteine transmethylase (Guest *et al.*, 1964*b*), whereas the vitamin B_{12}-dependent transmethylase is present only as apoenzyme. Cell-free extracts of *B. subtilis* grown in minimal medium supported methionine synthesis by extracts of *E. coli* 121/176 from serine and homocysteine when incubated together (Table 1). However, after removal of endogenous folates by passage of the extracts through Dowex 1 resin, the transmethylase in *B. subtilis* was active with $H_4PteGlu_3$ or preparation EHC as folate source but much less so with $H_4PteGlu_1$.

5-Methyltetrahydropteroyltriglutamate–homocysteine transmethylase was also measured directly by incubation of *B. subtilis* extracts with 5-$^{14}CH_3$-$H_4PteGlu_3$ and homocysteine as substrates under aerobic conditions. Such incubations measure the activity of a homocysteine transmethylase with properties similar to those of the vitamin B_{12}-independent enzyme of *E. coli* but not the vitamin B_{12}-dependent transmethylase (Dawes & Foster, 1971). *B. subtilis* extracts catalysed the production of labelled methionine from 5-$^{14}CH_3$-$H_4PteGlu_3$ by a reaction requiring homocysteine (Table 2). *S*-Adenosylhomocysteine did not replace homocysteine in these incubations and the addition of unlabelled *S*-adenosylmethionine in excess over the folate substrate did not alter the incorporation of [^{14}C]methyl groups into methionine, suggesting that *S*-adenosylmethionine is not a free intermediate in the reaction catalysed by *B. subtilis* 5-methyltetrahydropteroyltriglutamate–homocysteine transmethylase. Growth of the organism with added vitamin B_{12} did not affect the activity of this enzyme in extracts, whereas an exogenous supply of methionine considerably depressed the transmethylase activity.

When ultrasonic extracts of *B. subtilis* grown with added vitamin B_{12} were incubated anaerobically with 5-CH_3-$H_4PteGlu_3$ together with the cofactors required by the *E. coli* vitamin B_{12}-dependent transmethylase, the production of methionine was increased by about 15% (Table 2). However, this is likely to be of little significance since aerobic incuba-

Table 2. *5-$^{14}CH_3$-$H_4PteGlu_3$–homocysteine transmethylase in extracts of B. subtilis*

Ultrasonic extracts (5mg of protein/ml of reaction mixture) were incubated for 1h at 37°C under H_2. In experiment 1, the reaction mixture, M2, contained only the substrates and Mg^{2+} ions. *S*-Adenosylmethionine (8mM) and *S*-adenosylhomocysteine (5mM) were added where shown. In experiment 2, the reaction mixture, M2, was supplemented with *S*-adenosylmethionine (20μM), the reducing system (H) and methylcobalamin (10μM). $H_4PteGlu_1$ (6mM) and 5-CH_3-$H_4PteGlu_1$ (1.3mM) were added where shown. Growth supplements were cyanocobalamin, 2.5μg/l, and DL-methionine, 2mM.

Growth supplement	Addition	Omission	[*Me*-^{14}C]methionine formed (nmol/mg of protein) when incubated under: Air	H_2
Experiment 1				
None	None	None	74	
	None	Homocysteine	2	
	S-Adenosylhomocysteine	Homocysteine	3	
	S-Adenosylmethionine	None	74	
Cyanocobalamin	None	None	74	
Methionine	None	None	10	
Experiment 2				
Cyanocobalamin	None	None		87
	None	*S*-Adenosylmethionine, (H), methylcobalamin		89
	$H_4PteGlu_1$	None		98
	5-CH_3-$H_4PteGlu_1$	None		99

tion of crude preparations of *E. coli* vitamin B_{12}-dependent transmethylase gives only 1% of the activity found under anaerobic incubation conditions (Dawes, 1970). Further, omission of the presumed cofactors had no effect on the activity of *B. subtilis* extracts in anaerobic incubations.

There was no detectable inhibition of the conversion of 5-CH_3-H_4PteGlu$_3$ into methionine catalysed by *B. subtilis* extract by either H_4PteGlu$_1$ or 5-CH_3-H_4PteGlu$_1$.

5-Methyltetrahydropteroylmonoglutamate–homocysteine transmethylase

When cobalamin is added either to the growth medium or to ultrasonic extracts of *E. coli*, the extracts form methionine from 5-CH_3-H_4PteGlu$_1$ and homocysteine when incubated anaerobically together with *S*-adenosylmethionine and a reducing system (Weissbach *et al.*, 1963; Foster *et al.*, 1964*a*). Extracts of *B. subtilis* grown with added vitamin B_{12} were unable to use this folate for transmethylation under similar conditions, implying the absence of a vitamin B_{12}-dependent homocysteine transmethylase in *B. subtilis*. Further, added H_4PteGlu$_3$ did not promote the appearance of labelled methionine, showing that there is no enzyme capable of mediating transfer of a methyl group from 5-CH_3-H_4PteGlu$_1$ to H_4PteGlu$_3$ in *B. subtilis*.

These results account for the specificity for a polyglutamate folate coenzyme shown by *B. subtilis* extracts in the overall transfer of C_1 units from serine to homocysteine.

Methylcobalamin–homocysteine transmethylase

A further property of *E. coli* and other vitamin B_{12}-dependent transmethylases is their ability to use methylcobalamin as a substrate for transmethylation to homocysteine (Guest *et al.*, 1962; Weissbach *et al.*, 1963; Cauthen *et al.*, 1967). A slow, spontaneous methylation occurs in the absence of enzymic extract (Guest *et al.*, 1962). An extract of *E. coli* 3/62 increased this reaction four- to five-fold, but twice the amount of protein from *B. subtilis* had no comparable effect, even after growth of this organism with cyanocobalamin.

S-Adenosylmethionine as a methyl donor in methionine synthesis

Transmethylation from *S*-adenosylmethionine to homocysteine has been reported with extracts of *A. aerogenes*, *Saccharomyces cerevisiae*, *E. coli* K12 and other organisms (Shapiro, 1958). Even so, the net synthesis of methionine via this reaction would require the product, *S*-adenosylhomocysteine, to become remethylated. The conversion of *S*-adenosylmethionine into less cationic products (such as methionine) on incubation with homocysteine and *B. subtilis* extract was directly proportional to the *S*-adenosylmethionine concentration up to 5mM, implying that the reaction is non-enzymic or that the capacity of the enzyme to bind the substrate is low. In fact, the reaction proceeded in the absence of extract and was independent of homocysteine. Further tests showed that *S*-adenosylmethionine was not a methyl donor to tetrahydrofolates in the presence or absence of methylcobalamin, since tetrahydrofolates did not increase disappearance of *S*-adenosylmethionine from incubation mixtures with *B. subtilis* extract.

Occurrence of folates in B. subtilis

Folate content of B. subtilis. The organism was taken from mid-exponential growth phase (0.25mg dry wt./ml) for these assays. In lysozyme extracts assayed without further treatment, there was the equivalent of 0.51nmol of PteGlu$_1$/mg dry wt. of organism, detectable by *Lb. casei*. A similar value (equivalent to 0.50nmol of PteGlu$_1$/mg dry wt. of organism) was obtained by assay of the soluble extract derived by heating a washed suspension of *B. subtilis* at 100°C for 5min in potassium phosphate buffer, pH7.5 (20mM), containing 2-mercaptoethanol (15mM), and centrifuging (3900*g*; 20min).

Isolation of folates from B. subtilis. Since *B. subtilis* synthesizes methionine by a route involving an enzyme specific for a polyglutamate folate, some proportion of the native folates in this organism should be in a conjugated form. This was confirmed by the use of a simple procedure for the isolation and characterization of folate derivatives from bacterial extracts.

In the isolation of folates, autolysis of the extracts was avoided, since drastic alterations may well have occurred in the chemical structure of the native folates, as had been encountered in studies with mammalian liver (Chang, 1953; Donaldson & Keresztesy, 1959; Bird *et al.*, 1965).

Cell-free extracts of *B. subtilis* were prepared in buffer solutions containing 2-mercaptoethanol either by ultrasonication or by lysozyme treatment. The supernatant fluids after centrifuging at 0°C (25000*g*; 20min) were taken for gel filtration through Sephadex G-25 (Fig. 1). The columns were eluted with 20mM-potassium phosphate buffer, pH7.5, containing 15mM-2-mercaptoethanol. Fractions were monitored for folate by testing samples (0.1ml) for growth response by *Lb. casei*. Readings of E_{280} were also recorded to detect protein and other u.v.-absorbing material. Fractions with growth activity for *Lb. casei* which were eluted behind the peak of excluded material and which together contained more than 80% of the total extracted folate were pooled for the

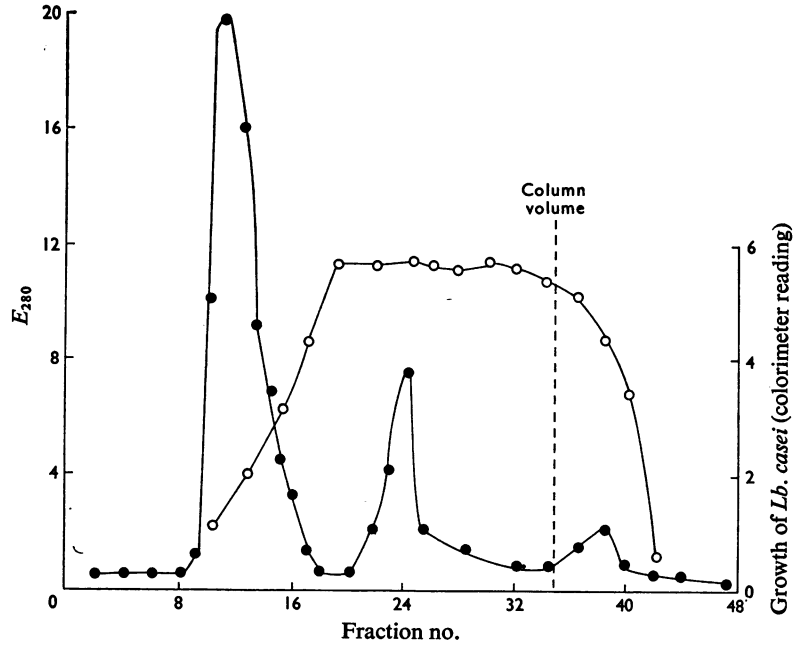

Fig. 1. *Gel filtration of a lysozyme extract of B. subtilis through Sephadex G-25*

The extract (10ml) was eluted from a column (52cm×9.6cm²) with 20mM-potassium phosphate buffer, pH7.5, containing 2-mercaptoethanol (15mM). Fractions (15ml) were collected. Samples (0.1ml) were tested for growth activity for *Lb. casei* (○). E_{280} (●) was measured against a blank containing 2-mercaptoethanol.

separation of folate derivatives by column chromatography on TEAE-cellulose. Folates were eluted from this column with a concentration gradient of ammonium acetate, pH6.1 (0–3M), containing 15mM-2-mercaptoethanol (Fig. 2). Fractions (3ml) were monitored for the presence and nature of folates by t.l.c. on cellulose and bioautography with *Lb. casei*. The NH_4^+ content of eluted fractions was estimated by assay with alkaline phenate (Russell, 1944).

All consecutive fractions containing a folate derivative with a particular R_F value were pooled and concentrated by freeze-drying, which also removed ammonium acetate. The solid residues were redissolved in 50mM-potassium phosphate buffer, pH7.0, containing 15mM-2-mercaptoethanol, for further tests. Spectrophotometric examination at 280nm of fractions eluted from the TEAE-cellulose column (Fig. 2) showed that about 90% of the u.v.-absorbing material was eluted before folates active for the growth of *Lb. casei* (fractions I and II).

Identification of isolated folates from B. subtilis extracted by lysozyme. Two chromatographically distinct folates were observed in the fractions eluted from TEAE-cellulose (I and II, Fig. 2). T.l.c. of fractions 35–42 revealed material (R_F0.82–0.86) active for the growth of *Lb. casei*. The absence of multiple or distorted growth zones on these bioautograms indicated that folate I was probably a single folate, the variation in R_F value being due to ammonium acetate present in the samples for t.l.c. A second folate, fraction II, R_F 0.47, was present in fractions 73–93. Folates I and II were each isolated by pooling the appropriate fractions and ammonium acetate was removed by freeze-drying. A sample of each folate was treated with a chicken pancreas conjugase preparation. T.l.c. of treated and untreated samples indicated that folates I and II were both polyglutamate folates (Table 3). Each isolated folate supported the growth of *Lb. casei*, and folate I was weakly active for the growth of *S. faecalis*. Only folate I satisfied the growth requirement of *P. pentosaceus*.

In solvent A (see the Materials and Methods section), the R_F of folate I without conjugase treatment (0.92) was close to the values for 10-HCO-H_4PteGlu$_3$ (R_F0.92), 5-HCO-H_4PteGlu$_3$ (R_F0.91) and 5-CH_3-H_4PteGlu$_3$ (R_F0.90). The chromatographic properties of folate I were unchanged by frequent exposure of an aqueous solution to air, indicating that folate I is not a labile 10-substituted tetrahydrofolate (Huennekens & Osborn, 1959). Further chromatographic evidence which demonstrated similarities

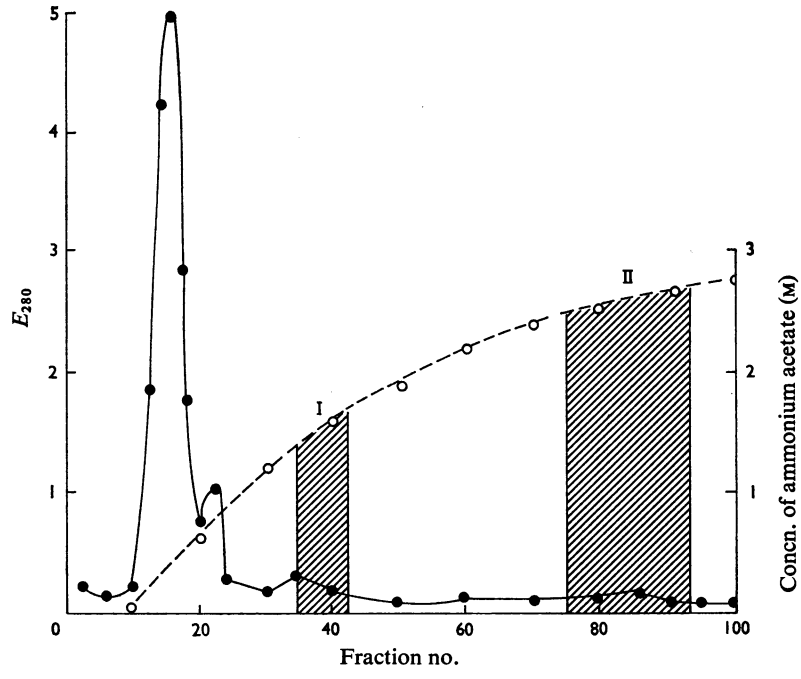

Fig. 2. *Elution of B. subtilis folates from TEAE-cellulose*

Folates were eluted from a column (25cm × 1.8cm²) of TEAE-cellulose (OH^- form) previously equilibrated with 15mM-2-mercaptoethanol, with a gradient of ammonium acetate, pH6.1 (0–3M), containing 15mM-2-mercaptoethanol (○). Fractions (3ml) were monitored for material absorbing at 280nm (●) and for folates by t.l.c. and bioautography with *Lb. casei*. The two species indicated by the hatched areas were distinguishable by their R_F values (see the text).

between folate I and 5-HCO-H_4PteGlu$_3$ rather than 5-CH_3-H_4PteGlu$_3$ is summarized below.

(*a*) 5-HCO-H_4PteGlu$_3$ is eluted from a TEAE-cellulose column at 1.3–1.6M-ammonium acetate, pH6.1, which is within the range for elution of folate I from a similar column (see Fig. 2 and Table 3), whereas 5-CH_3-H_4PteGlu$_3$ is eluted at only 0.53M-ammonium acetate (Guest *et al.*, 1964*a*).

(*b*) Folate I and 5-HCO-H_4PteGlu$_3$ behaved identically (R_F0.84) on t.l.c. with solvent B (see the Materials and Methods section), whereas 5-CH_3-H_4PteGlu$_3$ (R_F0.89) was readily distinguishable by two-dimensional chromatography.

(*c*) Folate I also co-chromatographed with 5-HCO-H_4PteGlu$_3$ (R_F0.39) in solvent C, and was separated from 5-CH_3-H_4PteGlu$_3$ (R_F0.54).

(*d*) T.l.c. on DE41 ion-exchange cellulose with a concentration gradient of sodium chloride (0.20–0.75M) distinguished folate I (R_F0.25) from 5-CH_3-H_4PteGlu$_3$ (R_F0.34).

The identity of folate II from *B. subtilis* remains unknown. It is probably a di- or tri-glutamate folate, since it is active for the growth of *Lb. casei*, which does not respond to folates containing more than three glutamate residues. However, its behaviour in t.l.c. is unlike PteGlu$_2$, PteGlu$_3$, their tetrahydro derivatives or their methyl or formyl compounds. The possible identity of folate II with a dihydropteroyldi- or tri-glutamate is not excluded by this work.

Identical results were obtained when the isolation and identification procedures were repeated with ultrasonic extracts of *B. subtilis*, suggesting that the major naturally occurring folates in this organism are not susceptible to possible oxidation, which may occur in the sonicator vessel (Hughes & Cunningham, 1963). The same two folates were also detected in ultrasonic extracts of *B. subtilis* grown with added vitamin B_{12} (5μg/l).

Discussion

The ability of cell-free extracts of *B. subtilis* to methylate homocysteine with serine as C_1-group source (20nmol/h per mg of protein) is similar to that

Table 3. *Properties of folates isolated from B. subtilis*

The procedure for t.l.c. and bioautography with *Lb. casei* is detailed in the Materials and Methods section. Superscripts refer to the solvents A–C used for t.l.c. (see the Materials and Methods section). A chicken pancreas conjugase preparation was used for the degradation of folates. Samples of folates I and II, containing ten times the amounts giving maximum growth (+++) of *Lb. casei* were tested for growth activity with *S. faecalis* and *P. pentosaceus*; + and (+) represent approximately 20% and 5% of maximum growth respectively.

	Isolated compounds			
	I	II	5-HCO-H_4PteGlu$_3$	5-CH_3-H_4PteGlu$_3$
Ammonium acetate concentration for TEAE-cellulose column chromatography	1.30M	2.36M	1.30M	0.53M*
Behaviour on cellulose thin layers				
Before conjugase treatment (R_F^A)	0.92	0.45	0.91	0.90
(R_F^B)	0.84		0.84	0.89
(R_F^C)	0.39		0.39	0.54
After conjugase treatment (R_F^A)	0.71	0.32	0.68†	0.75†
Growth response of				
Lb. casei	+++	+++		
S. faecalis	(+)	—		
P. pentosaceus	+	—		

* Guest *et al.* (1964*a*).

† These values refer to the monoglutamate derivatives, which are formed under the conditions used for conjugase treatment.

reported by Guest *et al.* (1960) for *E. coli* (19nmol/h per mg of protein), an organism that also does not contain significant amounts of endogenous cobalamin. After removal of endogenous folates, *B. subtilis* extracts have a specific requirement for a polyglutamate folate in the synthesis of methionine from homocysteine and serine. An examination of the individual enzymic reactions in this pathway has shown that the final transmethylase is inactive with 5-CH_3-H_4PteGlu$_1$, whereas serine transhydroxymethylase and 5,10-methylenetetrahydrofolate reductase are each active with monoglutamate folates. The 5-CH_3-H_4PteGlu$_3$–homocysteine transmethylase in *B. subtilis* is active under aerobic conditions with only the substrates and Mg^{2+} ions in phosphate buffer, and therefore resembles the cobalamin-independent homocysteine transmethylase in *E. coli* (Guest *et al.*, 1964*b*).

In the search for a cobalamin-dependent methylation of homocysteine similar to the reaction in *E. coli* (Guest *et al.*, 1960; Foster *et al.*, 1964*a*), the evidence indicates that no such mechanism operates in *B. subtilis*. This strain of *B. subtilis*, when grown in minimal medium, contains only traces of cobalamin (less than 11ng/g dry wt. of organism), compared with 3ng/g dry wt. for *E. coli* (Foster *et al.*, 1964*b*). These quantities are probably of little significance, for *Salmonella typhimurium* contains 700–1100ng/g dry wt., yet this is insufficient to permit growth of cobalamin/methionine auxotrophs, which have lost the cobalamin-independent transmethylase activity by mutation (Cauthen *et al.*, 1966). Calculations (Cauthen, 1965) have shown that growth of cobalamin auxotrophs of *E. coli* ceases when the vitamin B_{12} content of the cells falls to that found as endogenous material in *S. typhimurium*. *B. subtilis* grown in minimal medium contains only traces of vitamin B_{12} (present as hydroxocobalamin), and the potential operation of a cobalamin-dependent methylation of homocysteine has therefore been examined by using extracts of *B. subtilis* grown with added cyanocobalamin. The presence of vitamin B_{12} had no effect on the growth of the organism.

After growth of *B. subtilis* with added cyanocobalamin, chromatographic separation of the cobamide derivatives from ethanolic extracts did not reveal any methylcobalamin. This derivative, reported to be an intermediate in the transmethylation to homocysteine from 5-methyltetrahydrofolate in *E. coli* (Taylor & Weissbach, 1968), was easily detected in ethanolic extract of *E. coli* PA 15 grown with added cyanocobalamin (Salem, 1970). Nevertheless, 5′-deoxyadenosylcobalamin was found in extracts of *B. subtilis* grown in the presence of cyanocobalamin. Thus this organism, which contains little or no endogenous cobalamin, is able to metabolize exogenously added vitamin B_{12} to produce a coenzyme which in other organisms is required for a number of reactions usually involving hydrogen transfer (Stadtman, 1971). The detection of any of

these reactions in *B. subtilis* induced or activated by growth with vitamin B_{12} would provide further examples of the existence of enzymes whose action is not essential to the growth of the organism and relies entirely on an exogenous source of the cofactor. Such an enzyme is the cobalamin-dependent homocysteine transmethylase of *E. coli*, the activity of which becomes essential for growth only when the vitamin B_{12}-independent mechanism is eliminated by mutation or where there is an exogenous supply of cobalamin (Foster *et al.*, 1964*b*).

The activity of the enzymes catalysing the generation and transfer of methyl groups is not altered by the growth of *B. subtilis* with added vitamin B_{12}. Thus unlike *E. coli*, the methylation of homocysteine by an ultrasonic extract of *B. subtilis* still requires a polyglutamate folate; 5-CH_3-H_4PteGlu$_1$ is an ineffective methyl donor even when all the cofactors required by the *E. coli* cobalamin-dependent enzyme are supplied. That growth with added cobalamin does not evoke a cobalamin-dependent transmethylase specific for 5-CH_3-H_4PteGlu$_3$ is strongly suggested by the finding that methyl transfer is still unaffected by air, by provision of an exogenous reducing system, by methylcobalamin or by *S*-adenosylmethionine. Moreover, growth with added cyanocobalamin did not repress the vitamin B_{12}-independent transmethylase as found for *E. coli* (Dawes & Foster, 1971).

The possibility that methionine may be produced from *S*-adenosylmethionine by transmethylation to homocysteine as reported by Shapiro (1958) for *A. aerogenes*, *S. cerevisiae* and *E. coli* K12 (but not *E. coli* strains B and W) or by transadenosylation as suggested for *E. coli* (Texas) by Pfeffer & Shapiro (1962) has been tested with *B. subtilis* extract. However, the conversion of *S*-adenosylmethionine into non-cationic products, e.g. methionine and *S*-adenosylhomocysteine, is non-enzymic and independent of homocysteine, unlike the activities reported for the other organisms. This slow breakdown of *S*-adenosylmethionine is not thought to be significant compared with the rate of methionine synthesis by *B. subtilis*. Pertinent to this, *S*-adenosylhomocysteine does not substitute for homocysteine as methyl acceptor from 5-CH_3-H_4PteGlu$_3$, yet it would be essential for *S*-adenosylhomocysteine somehow to become remethylated for net synthesis of methionine from *S*-adenosylmethionine. The failure of excess of unlabelled *S*-adenosylmethionine to decrease incorporation of radioactivity into methionine from 5-CH_3-H_4PteGlu$_3$ confirms that *S*-adenosylmethionine is not an intermediate between the folate and methionine. Further, *S*-adenosylmethionine does not serve as a methyl donor to homocysteine in the presence of tetrahydrofolate, indicating that *S*-adenosylmethionine is not a source of the methyl group of 5-CH_3-H_4PteGlu$_3$.

With this evidence against the existence of a cobalamin-dependent transmethylase in *B. subtilis*, it appears that the pathway to methionine from serine resembles the vitamin B_{12}-independent sequence established for *E. coli* PA15 (Woods *et al.*, 1965) involving serine transhydroxymethylase, 5,10-methylenetetrahydrofolate reductase and 5-methyltetrahydropteroyltriglutamate–homocysteine transmethylase. *B. subtilis* therefore depends on conjugated folates for methionine biosynthesis. Both the overall C_1-group transfer and 5-CH_3-H_4PteGlu$_3$–homocysteine transmethylase activity are depressed by growth of the organism with methionine.

The isolation of naturally occurring folates and their characterization by comparison with known synthetic derivatives has revealed only polyglutamate derivatives in *B. subtilis*. This is in accordance with the specific requirement for a conjugated folate by the homocysteine transmethylase. 5-CH_3-H_4PteGlu$_3$ was not found, but one of the two folates isolated was 5-HCO-H_4PteGlu$_3$, a derivative found previously in this organism by Hakala & Welch (1957). The structure of the other was not established, but may be a derivative of PteGlu$_2$ or its dihydro or tetrahydro forms. The occurrence of other, higher conjugates is not precluded, since these would not have been detected directly by assay with *Lb. casei*.

The occurrence of only polyglutamate folates in *B. subtilis* indicates that the general metabolic reactions of folates normally operate with conjugated forms, even though some of the enzymes are active *in vitro* with the corresponding monoglutamate derivatives.

The finding of a cobalamin-independent homocysteine transmethylase specific for a polyglutamate folate substrate and the absence of a vitamin B_{12}-dependent transmethylase are consistent with the inability of *B. subtilis* to synthesize cobalamin. The origin and function of the dual pathway to methionine in *E. coli*, however, remain obscure.

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