Vitamin B₁₂ in Photosynthetic Bacteria and Methionine Synthesis by *Rhodospseudomonas spheroides*

By SALLY E. CAUTHEN,* J. R. PATTISON AND JUNE LASCELLES†
Microbiology Unit, Department of Biochemistry, University of Oxford

(Received 13 June 1966)

1. Assay of some photosynthetic bacteria for vitamin B₁₂ showed them to be relatively rich in this factor. *Rhodospseudomonas spheroides*, grown photosynthetically in Co²⁺-supplemented medium, contained about 100 µg./g. dry wt.
2. Extracts of wild-type *Rps. spheroides* methylated homocysteine by a mechanism similar to the cobalamin-dependent pathway present in *Escherichia coli*. However, no mechanism similar to the cobalamin-independent N⁵-methyltetrahydrofolate-homocysteine transmethylase of *E. coli* could be detected in *Rps. spheroides*.
3. N⁵N¹⁰-Methylenetetrahydrofolate-reductase activity was found in *Rps. spheroides*.
4. A methionine-requiring mutant strain of *Rps. spheroides* (strain 2/33), which does not respond to homocysteine, made the same amount of vitamin B₁₂ as the parent organism. Extracts did not form methionine from N⁵-methyltetrahydrofolate and homocysteine even in the presence of cofactors shown to be necessary with the parent strain, and it is concluded that the mutant is blocked in the formation of the apoenzyme of a homocysteine-methylating system similar to the vitamin B₁₂-dependent one in *E. coli*.

Escherichia coli strains PA15 and 121-176 do not produce vitamin B₁₂; when grown in an environment devoid of the vitamin, these strains are independent of cobalamin in the methylation of homocysteine by conjugated forms of 5-CH₃-H₄-PteGlu† (Foster, Tejerina, Guest & Woods, 1964b). (In the present paper the terms folate, vitamin B₁₂ and cobalamin are used in general reference to the two families of compounds; names in common use will be used in reference to specific members of these families, e.g. tetrahydropteroylglutamate and methylcobalamin.) However, *E. coli* produces an apoenzyme that, in the presence of vitamin B₁₂, either in the growth medium or supplied exogenously to the cell-free extracts, catalyses the transfer of methyl groups from 5-CH₃-H₄PteGlu to homocysteine (Kisliuk, 1960; Foster, Jones & Woods, 1961). The latter mechanism is dependent on the additional cofactors SAM and FADH₂, and is not specific with respect to the form of folate

substrate (Foster, Dilworth & Woods, 1964a; Guest, Friedman, Foster, Tejerina & Woods, 1964b). Thus alternative pathways for the methylation of homocysteine are present in this organism (Foster *et al.* 1964b; Guest, Foster & Woods, 1964a; Guest *et al.* 1964b).

Similarly, alternative pathways for the transfer of methyl groups from methylated tetrahydrofolates to homocysteine have been described in *Salmonella typhimurium* (Cauthen, Foster & Woods, 1966) and in *Aerobacter aerogenes* (Morningstar & Kisliuk, 1965).

An enzyme catalysing the transfer of a methyl group from N⁵-methyltetrahydrofolate to homocysteine has been purified 240-fold from hog liver by Loughlin, Elford & Buchanan (1964). It contains cobalamin and requires FADH₂ as well as SAM.

Dickerman, Redfield, Bieri & Weissbach (1964) reported the 30-fold purification of N⁵-methyltetrahydrofolate-homocysteine transmethylase from chicken liver. A transfer of the methyl group from methylcobalamin to homocysteine was demonstrated after the observation of this reaction in *E. coli* by Guest, Friedman, Woods & Smith (1962). Only enzymes and homocysteine were required; there was no effect of SAM or 5-CH₃-H₄PteGlu.

The object of the present work was to provide evidence at the enzymic level for the mechanism of N⁵-methyltetrahydrofolate-homocysteine

* Present address: Department of Chemistry, Abilene Christian College, Abilene, Texas, U.S.A.

† Present address: Department of Bacteriology, University of California, Los Angeles, Calif., U.S.A.

‡ Abbreviations: for members of the folate group, based on PteGlu for pteroylglutamate; H₄PteGlu, tetrahydropteroylmonoglutamate; H₄PteGlu₃, tetrahydropteroyltriglutamate; derivatives of these compounds are indicated as, e.g., 5-CH₃-H₄PteGlu for N⁵-methyltetrahydropteroylmonoglutamate; SAM, S-adenosylmethionine.

methyl transfer in *Rhodospseudomonas spheroides*. This followed the isolation of a mutant of *Rps. spheroides* that responded to methionine but not to homocysteine or to vitamin B₁₂ (Lascelles, 1966), and which was therefore presumably blocked in this transmethylation. Since vitamin B₁₂ was found in relatively high concentration in this organism a survey of other photosynthetic bacteria was also made as a corollary to this work.

MATERIALS AND METHODS

Organisms. The strains of Athiorhodaceae were: *Rps. spheroides* N.C.I.B. 8253 and the methionine-requiring auxotroph, 2/33, derived from it (Lascelles, 1966); *Rhodospseudomonas capsulata* van Niel strain 2.3.11 and that described by Cooper (1963); *Rhodospirillum rubrum*, obtained from Dr R. Y. Stanier. These organisms were maintained as stab cultures on yeast extract-malate-glutamate medium (Lascelles, 1956). The green sulphur photosynthetic bacteria were *Chlorobium thiosulphatophilum* N.C.I.B. 8327 and N.C.I.B. 8346. These were maintained as stab cultures on thiosulphate-bicarbonate medium as described by Hurlbert & Lascelles (1963), except that 0.2% (w/v) NaHCO₃ was used and the pH was 6.8–7.0.

E. coli strain 121/176 is an auxotroph derived from the prototrophic strain W (A.T.C.C. 9637) and requires either methionine or cobalamin for growth (Davis & Mingioli, 1950). *E. coli* strain 3/62 requires methionine for growth and does not respond to either cobalamin or homocysteine; it was isolated from *E. coli* 518 (A.T.C.C. 9723) in this Laboratory by the technique of Adelberg & Meyers (1953). Strain 121/176 lacks N⁵-methyltetrahydropteroyltriglutamate-homocysteine transmethylase and strain 3/62 lacks N⁵N¹⁰-methylene tetrahydrofolate reductase (Foster *et al.* 1964b).

Growth of organisms and preparation of suspensions. For Athiorhodaceae the malate-glutamate medium of Lascelles (1959) was used, supplemented with 4 μM-CoCl₂; Difco casein hydrolysate ('casamino acids') or methionine was used as noted in the text. In some experiments, malate and glutamate were replaced by sodium propionate (10 mM) plus NaHCO₃ (6 mM). Cultures were grown anaerobically in the light or in the dark with high aeration (Lascelles, 1956, 1959). They were harvested by centrifugation at the end of the exponential phase, when the cell density had reached 0.7–0.9 mg. dry wt./ml. The cells were washed in one-half the original culture volume of 40 mM-potassium phosphate buffer, pH 7.5. For determination of cobalamin content, the washed cells were resuspended in water to give about 10 mg. dry wt./ml. For enzymic work the washed cells were resuspended in 50 mM-potassium phosphate buffer, pH 7.5, containing mercaptoethanol (7 mM), to a density of about 20 mg. dry wt./ml.

The *Chlorobium* (green sulphur photosynthetic bacteria) strains were grown on the thiosulphate-bicarbonate medium of Hurlbert & Lascelles (1963) modified to contain 24 mM-NaHCO₃ and with the pH adjusted to 6.8–7.0. The organisms were grown anaerobically in the light in completely filled bottles as described by Hurlbert & Lascelles (1963). They were harvested after 3 days at the end of the exponential phase. Cultures were harvested and suspensions prepared as described above.

E. coli organisms were grown on solid defined media in enamelled trays as described by Guest, Helleiner, Cross & Woods (1960). The basal medium was supplemented with DL-methionine (0.5 mM). Where indicated, cobalamin (15 μmoles/500 ml. of medium) was used as a supplement, alternative to methionine.

Extraction and assay of cobalamin. After release of the vitamin by heating in the presence of KCN and subsequent papain digestion, the supernatant fluids, after centrifuging, were assayed for cyanocobalamin with *Euglena gracilis* var. *bacillaris* by the procedure of Foster *et al.* (1964b). Alternatively a more rapid, though less sensitive, procedure of assay with *E. coli* 113/3 was used (Foster *et al.* 1961); in this case the vitamin was released by heating at 100° in the presence of KCN as described by Ford, Holdsworth & Kon (1955).

Cell-free extracts. Enzyme extracts of *Rps. spheroides* were prepared by ultrasonic oscillation (Lascelles, 1959). *E. coli* extracts were prepared by the method of Guest *et al.* (1960) modified by the use of 20 mM-potassium phosphate buffer, pH 7.8, containing 2-mercaptoethanol (7 mM), for the preparation of suspensions of organisms before ultrasonic treatment. Extracts were stored at –20° and lost no activity over a period of 6 months.

The protein content of non-pigmented extracts was determined spectrophotometrically by the method of Layne (1957). Protein concentration in pigmented organisms was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). At the concentrations involved the pigments did not interfere with the determination.

An extract of heated *E. coli* (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 H₄PteGlu₃ as a cofactor in methionine synthesis by enzymic extracts of *E. coli*.

Tests for methionine synthesis. The conditions used for testing methionine synthesis were based on previous work with *E. coli* in this Laboratory. When serine was the ultimate source of C₁ units, the basal reaction mixture, M1, contained in a final volume of 1 or 2 ml.: potassium phosphate buffer (pH 7.8), 120 mM; DL-homocysteine, 6–7 mM; L-serine, 5 mM; ATP, 5 mM; glucose, 20 mM; MgSO₄, 5 mM; NAD, 0.5 mM; FMN, 0.25 mM; pyridoxal phosphate, 0.5 mM. Enzyme preparation was added equivalent to 1–10 mg. of protein/ml. The source of folate cofactor was extract of heated *E. coli*; the equivalent of 14 mg. dry wt. of original organisms was used/ml. of reaction mixture.

The complete reaction mixtures were incubated at 37° for 1 hr. in an atmosphere of H₂ and the enzymic reaction was terminated by heating for 3 min. at 100°. The supernatant fluids, after centrifuging, were used for the assay of methionine by the microbiological method of Gibson & Woods (1960). DL-Methionine was used as standard; the organism responds only to the L-isomer. The value obtained (15–20 μmoles) in each test for a control containing heated enzyme has been deducted from the experimental values recorded. No methionine degradation occurs under the incubation conditions since added L-methionine was completely recovered.

When N⁵[Me-¹⁴C]-methyltetrahydrofolate was the source of C₁ units the incubation mixture, M2, contained in a final volume of 0.55 ml.: potassium phosphate buffer (pH 7.8),

100mM; DL-homocysteine, 24mM; 2-mercaptoethanol, 21.3mM; MgSO_4 , 5mM. Other additions made were: SAM, 20 μM ; ATP, 20mM; a reducing system [H] consisting of FAD (75m μmoles), NAD (250m μmoles), ethanol (54 μmoles) and alcohol dehydrogenase (90 μg .); methylcobalamin, 10 μM ; DL-methionine, 2mM. Enzyme preparation equivalent to 2.5mg. of protein was added. Substrates were either $(\pm)$ -[Me- ^{14}C]5-CH $_3$ -H $_4$ PteGlu, 1.1moles (0.37 μC), or $(\pm)$ -[Me- ^{14}C]5-CH $_3$ -H $_4$ PteGlu $_3$, 1.6moles (0.8 μC), per incubation volume.

The reaction mixtures were incubated at 37° in an atmosphere of H $_2$ for between 15 and 120min. The incubated reaction mixtures were passed through columns of Dowex 1 resin (X8; Cl $^-$ form; 100–200 mesh) to remove unchanged folate as described by Foster *et al.* (1964a). After the columns had been washed twice with 1ml. of water, samples (0.2ml.) of the combined effluent and washings were dissolved in 7ml. of scintillation fluid containing 0.4% (w/v) *p*-terphenyl and 0.07% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene-ethanol-dioxan (12:5:3, by vol.). Radioactivity was estimated at 70% efficiency with an automatic scintillation counter (type 6020; Isotope Developments Ltd., Reading, Berks.). None of the materials present caused significant quenching of [Me- ^{14}C]methionine. Methionine was the only radioactive product detected radioautographically after descending paper chromatography on Whatman no. 1 paper with as solvent butan-1-ol-acetic acid-water (4:1:5, by vol.; upper layer) or 2,4,6-collidine-2,5-lutidine-water (1:1:2, by vol.; upper layer). The amount of L-methionine formed was calculated from the initial specific activity of the N ^{5} -methyltetrahydrofolate and agreed with values obtained by occasional random microbiological assays.

Methylcobalamin-homocysteine transmethyase was assayed as described by Guest *et al.* (1962), by incubating enzymic extracts with methylcobalamin and homocysteine in phosphate buffer and estimating the resulting methionine microbiologically.

Tests for the synthesis and degradation of S-adenosyl-methionine. From previous work in this Laboratory the breakdown of SAM was followed by assessing the radioactivity of incubated mixtures after the removal of any intact [Me- ^{14}C]SAM. The basal reaction mixture M4 contained in a final volume of 0.5ml.: potassium phosphate buffer (pH 7.8), 100mM; [Me- ^{14}C]SAM, 150 μM ; DL-homocysteine, 6.7mM; the reducing system [H] as described above. Two different amounts of enzyme preparation were added and the complete reaction mixtures were incubated at 37° in an atmosphere of H $_2$ for 2hr. After incubation the mixtures were passed through columns of Dowex 50 resin (X8; Li $^+$ form; 200–400 mesh) to remove any intact [Me- ^{14}C]SAM. After washing the columns twice with 1ml. of water the radioactivity of samples (0.2ml.) was measured as described above.

Also based on previous work in this Laboratory the synthesis of SAM was measured by following the conversion of L-[Me- ^{14}C]methionine and ATP into SAM in the presence of Mg $^{2+}$. The basal reaction mixture M3 contained in a final volume of 0.5ml.: potassium phosphate buffer (pH 7.8), 100mM; 2-mercaptoethanol, 25mM; MgSO_4 , 5mM; ATP, 20mM; L-[Me- ^{14}C]methionine, 1mM. Enzyme preparation equivalent to 3mg. of protein was added and the complete reaction mixtures were incubated at 37° in an atmosphere of H $_2$ for 2hr. After the addition of 5 μmoles of SAM as

carrier the incubated reaction mixtures were passed through columns of Dowex 1 resin (Cl $^-$ form; X8; 100–200 mesh) to remove unchanged ATP. The columns were washed twice with 1ml. of water, the combined effluent and washings being collected in tubes containing 0.5ml. of 60% (w/v) HClO $_4$. After centrifuging, the supernatants were passed through columns of Dowex 50 resin (Li $^+$ form; X8; 200–400 mesh) to remove any SAM formed. After the columns had been washed with 20ml. of 2N-HCl, the SAM was eluted in two fractions each consisting of 3 $\times$ 1.0ml. of 6N-HCl. The radioactivity of samples (0.2ml.) of these eluates was measured as described above. After appropriate dilution with water the extinction of the eluate at 256m μ was measured in a Unicam SP.500 spectrophotometer. The amount of radioactive SAM formed during incubation was calculated from the measurement and the known amount of carrier SAM added before column treatment.

Chemicals. $(\pm)$ -N ^{5} [Me- ^{14}C]-Methyltetrahydrofolates were prepared by reduction of $(\pm)$ -N ^{5} N ^{10} [^{14}C]-methylene-tetrahydrofolates and purified by column chromatography as described by Guest *et al.* (1964a). Solutions in potassium phosphate buffer, pH 7.5 (50mM), containing 2-mercaptoethanol (7mM), were standardized spectrophotometrically (by using ϵ 28000M $^{-1}$ cm. $^{-1}$ at 290m μ) and stored under H $_2$ at -20°. SAM was isolated from yeast by the method of Schlenk, Dainko & Stanford (1959). Methylcobalamin was a gift from Dr E. Lester Smith; crystallized cyanocobalamin was from British Drug Houses Ltd. (Poole, Dorset). Crystallized yeast alcohol dehydrogenase was from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). ATP, NAD, FMN and FAD were products of the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Pyridoxal phosphate (Roche Products Ltd., Welwyn Garden City, Herts.) and DL-homocysteine (Mann Research Laboratories Inc., New York, N.Y., U.S.A.) were also commercial products.

RESULTS

Synthesis of cobalamin by photosynthetic bacteria. *Rps. spheroides* 8253 (wild-type), grown either aerobically in the dark or anaerobically in the light, synthesized cobalamin when grown on malate-glutamate medium unsupplemented with Co $^{2+}$ (Table 1). The cobalamin content of the cells was considerably increased by addition of cobalt chloride (4 μM) to the medium, particularly in photosynthetic cultures. The methionine-requiring mutant strain 2/33 formed amounts of vitamin B $_{12}$ comparable with the parent strain when grown under similar conditions.

The other species of *Rhodospseudomonas* tested, namely *Rps. capsulata* strains 2.3.11 and Cooper, likewise were competent for synthesis of the vitamin even when grown in the absence of Co $^{2+}$.

Rsp. rubrum synthesized rather smaller but significant amounts of cyanocobalamin. No difference in the ability to synthesize the vitamin was observed when propionate was substituted for malate as the carbon source in the medium.

Cyanocobalamin contents of two strains of

Table 1. Cobalamin content of photosynthetic bacteria

Details of the growth and assay conditions are given in the Materials and Methods section. The various media were supplemented with CoCl_2 as shown. M-G medium, malate-glutamate medium.

Organism	Growth conditions		CoCl_2 ($4\mu\text{M}$)	Cyanocobalamin ($\mu\text{g./g. dry wt. cells}$)
<i>Rps. spheroides</i> 8253	M-G medium	Aerobic	—	8.6
		Aerobic	+	20
		Anaerobic (in light)	—	9.5
		Anaerobic (in light)	+	102
<i>Rps. spheroides</i> 2/33	M-G medium + L-methionine (0.1 mM)	Aerobic	—	9.3
<i>Rps. capsulata</i> 2.3.11	M-G medium	Anaerobic (in light)	+	46
<i>Rps. capsulata</i> Cooper	M-G medium + casein hydrolysate (0.1%)	Anaerobic (in light)	+	67
<i>Rps. rubrum</i>	M-G medium + casein hydrolysate (0.1%)	Anaerobic (in light)	+	4.6
<i>Rps. rubrum</i>	Propionate- CO_2 medium + casein hydrolysate (0.1%)	Anaerobic (in light)	+	4.3
<i>C. thiosulphatophilum</i> 8327	Thiosulphate- CO_2 medium	Anaerobic (in light)	+	35
<i>C. thiosulphatophilum</i> 8346	Thiosulphate- CO_2 medium	Anaerobic (in light)	+	40

Table 2. Methylene-tetrahydrofolate reductase and methyl-tetrahydrofolate-homocysteine transmethyase in *Rps. spheroides* 8253 and 2/33

Organisms were grown photosynthetically (Expt. 1) or aerobically (Expt. 2) in malate-glutamate medium (M-G medium) supplemented with CoCl_2 or L-methionine as shown. Extracts (3 mg. of protein) were incubated for 1 hr. at 32° (Expt. 1) or 37° (Expt. 2) in mixture M1 with added *E. coli* extract (3 mg. of protein) as shown.

Expt. no.	Strain of <i>Rps. spheroides</i>	Supplement to M-G medium	L-Methionine formed (μmoles) with <i>E. coli</i> extract*		
			Nil	3/62	121/176
1	8253	None	0	400	12
	8253	CoCl_2 ($4\mu\text{M}$)	6	150	65
2	8253	None	0	440	78
	8253	Methionine (0.1 mM)	0	154	36
	2/33	Methionine (0.1 mM)	0	900	60

* Methionine formation by either *E. coli* extract alone was slight (about $20\mu\text{moles}$); when mixed together, the *E. coli* extracts formed 600 and $500\mu\text{moles}$ in Expts. 1 and 2 respectively.

sulphur bacteria, *C. thiosulphatophilum* 8327 and 8346, were similar to those found in *Rps. capsulata*.

Overall formation of methionine from homocysteine and serine by extracts of *Rps. spheroides*. When ultrasonic extracts of *Rps. spheroides* 8253, grown either with or without Co^{2+} , were incubated in reaction mixture M1 no significant synthesis of methionine from serine could be detected (Table 2, Expt. 1). However, inclusion of SAM ($20\mu\text{M}$) in the reaction mixture resulted in rapid methionine formation ($430\mu\text{moles/3mg. of protein/hr.}$) by these same extracts. Thus there is an absolute requirement for SAM even in the presence of the relatively complex incubation mixture M1.

N^5N^{10} -Methylene-tetrahydrofolate reductase of *Rps.*

spheroides. The amount of this enzyme in an extract can be assessed by the extent to which the extract promotes the synthesis of methionine from serine by ultrasonic extracts of *E. coli* strain 3/62, since the enzyme is specifically lacking in this strain (Foster *et al.* 1964b; Guest *et al.* 1964a).

By using this test, reductase activity was readily detected in extracts of strain 8253 (Table 2, Expt. 1). Extracts of organisms grown in the presence of Co^{2+} had apparently less than half as much reductase as those of organisms grown in the absence of added metal ion. As growth in the presence of Co^{2+} substantially increases the cobalamin content of *Rps. spheroides* 8253 (Table 1), this result is in accord with results obtained with *E. coli* in which

the amount of this enzyme is similarly decreased in organisms grown in the presence of vitamin B₁₂ (Dr M. A. Foster, personal communication).

A similar decrease in the reductase was found when the organism was grown in the presence of methionine (Table 2, Expt. 2). In this case the wild-type strain 8253 was grown aerobically for more direct comparison with the mutant strain 2/33, which will not grow in the absence of oxygen. Since the addition of methionine to the growth medium apparently decreases the N⁵N¹⁰-methyl-enetetrahydrofolate reductase of the parent, extracts of the mutant strain (which must be grown with added methionine) were expected to contain a decreased amount of the enzyme. However, these

extracts contained over twice as much enzyme as extracts of strain 8253 grown without methionine (Table 2, Expt. 2). This finding has not been explained.

N⁵-Methyltetrahydrofolate-homocysteine trans-methylase of *Rps. spheroides*. *E. coli* strain 121/176 specifically lacks N⁵-methyltetrahydropteroyltriglutamate-homocysteine transmethylase; ultrasonic extracts of this organism grown with a methionine supplement can then synthesize methionine from homocysteine and serine with reaction mixture M1 only if provided with a N⁵-methyltetrahydrofolate-homocysteine transmethylase, such as the cobamide-containing enzyme of the N⁵-methyltetrahydropteroyltriglutamate-homocysteine transmethylase of *E. coli* (Foster *et al.* 1964b; Guest *et al.* 1964b).

Extracts of both wild-type and mutant *Rps. spheroides* had a weak ability to promote methionine synthesis by *E. coli* strain 121/176 (Table 2). This transmethylase activity was further examined with the defined N⁵-methyltetrahydrofolate substrates.

N⁵-Methyltetrahydropteroyltriglutamate-homocysteine transmethylase by *Rps. spheroides*. Extracts of *Rps. spheroides*, grown photosynthetically in malate-glutamate medium, when incubated in mixture M2 did not form methionine from homocysteine and 5-CH₃-H₄PteGlu in the absence of added cofactors (Table 3). Although the addition of SAM alone did not enhance this ability significantly, the addition of SAM and a reducing system did do so. The further addition of methylcobalamin did not alter the amount of methionine formed; S-adenosylhomocysteine did not replace SAM as a cofactor. Growth of the organism in the presence of Co²⁺ produced similar results.

Table 3. N⁵-Methyltetrahydropteroyltriglutamate-homocysteine transmethylase by *Rps. spheroides* 8253 grown with and without Co²⁺ ions

Rps. spheroides 8253 was grown photosynthetically in malate-glutamate medium with CoCl₂ (4 μM) as shown. Extracts (3 mg. of protein) were incubated for 1.5 hr. at 32° in mixture M2 with additions as shown. [H], Reducing system defined in the text.

Addition	L-Methionine formed (mμmoles) by extracts grown with	
	No supplement	Co ²⁺
None	0	0
SAM	34	60
SAM, [H]	439	460
SAM, [H], methylcobalamin	415	530
SAM, [H]	0	0

Table 4. Cofactor-dependent N⁵-methyltetrahydrofolate-homocysteine transmethylase in *Rps. spheroides* 8253 and 2/33

The organisms were grown aerobically in malate-glutamate medium (M-G medium), supplemented with L-methionine (0.1 mM) in the case of strain 2/33. In Expt. 1 strain 8253 was grown without methionine and in Expt. 2 with L-methionine (0.1 mM) as shown. Extracts (3 mg. of protein) were incubated for 1 hr. or for 2 hr. in Expts. 1 and 2 respectively at 37° in mixture M2 with either 5-CH₃-H₄PteGlu (Expt. 1) or 5-CH₃-H₄PteGlu₃ (Expt. 2) as substrate and with additions as shown. [H], Reducing system defined in the text.

Expt. no.	Supplement to M-G medium	Additions to mixture M2	L-Methionine formed (mμmoles) by extracts of strain	
			8253	2/33
1	None	None	0	0
		[H]	0	0
		SAM	84	0
		SAM, [H]	500	3
		SAM, [H], methylcobalamin	509	4
2	None	None	20	—
		SAM, [H], methylcobalamin	690	—
	Methionine	None	15	18
		SAM, [H], methylcobalamin	590	30

With extracts of strain 2/33, however, methionine synthesis with 5-CH₃-H₄PteGlu as methyl donor is barely detectable, even in the presence of cofactors shown to be required for methionine synthesis by extracts of strain 8253 (Table 4, Expt. 1).

*N*⁵-Methyltetrahydropteroyltriglutamate - homocysteine transmethylation by *Rps. spheroides*. Extracts of strain 8253 (grown with or without methionine) and strain 2/33 synthesize negligible amounts of methionine, in the absence of cofactors, with 5-CH₃-H₄PteGlu₃ as methyl donor (Table 4, Expt. 2). However, with strain 8253 the addition of the cofactors SAM, the reducing system [H] and methylcobalamin increases this synthesis some 30-fold, whereas with strain 2/33 it is without significant effect.

The results with strain 8253 make it unlikely that growth in the presence of methionine contributed significantly to the failure to detect any

synthesis of methionine by extracts of strain 2/33 even when the cofactors were provided.

Methylcobalamin-homocysteine transmethylase of *Rps. spheroides*. In *E. coli* the cobamide-containing *N*⁵-methyltetrahydrofolate-homocysteine transmethylase also catalyses the methylation of homocysteine by methylcobalamin (Guest *et al.* 1962). Since the results so far presented suggest that strain 2/33 lacks *N*⁵-methyltetrahydrofolate-homocysteine transmethylase, it was expected that its extracts would not catalyse methyl transfer from methylcobalamin to homocysteine. However, the mutant extracts did have this ability, showing the same activity as the parent strain (37 μmoles of L-methionine/mg. of protein/hr.).

S-Adenosylmethionine requirement. The requirement for SAM (Fig. 1) in the methylation of homocysteine by extracts of strain 8253 (calculated *K_m* 14.3 μM) is high compared with *E. coli* (*K_m* 0.65 μM; Foster *et al.* 1964a). Virtually no methionine synthesis was detected with extracts of strain 2/33 even with 50-fold the usual amount of SAM, at a final concentration of 1 mM. The lack of response of extracts of the mutant to high concentrations of SAM made it unlikely that its failure in methionine synthesis was due to inability to form SAM. This was confirmed by direct assay for SAM synthetase in extracts of strain 8253 and 2/33 (Table 5, Expt. 1). Although the enzyme was repressed by methionine in the growth medium, its activity in strain 2/33 was similar to that in the parent organism grown with the same amount of methionine.

The possibility that the high requirement of strain 8253 for SAM was due to its rapid degradation by the extracts was also examined. However, in both the parent and mutant, the rate of SAM degradation was no greater than its rate of synthesis (Table 5). Since in the parent strain (grown without methionine) SAM can be formed at a rate twice that of its degradation, the requirement for SAM in methionine synthesis by extracts of strain 8253 should be satisfied by a mixture of ATP, Mg²⁺ and L-methionine. Fig. 2 shows that this is so; the

Fig. 1. Effect of the concentration of SAM on the synthesis of methionine by extracts of *Rps. spheroides* strain 8253 (○) and 2/33 (●). Extracts (1.2 mg. of protein) were incubated for 15 min. at 37° in mixture M2 plus reducing system [H], with 5-CH₃-H₄PteGlu as substrate.

Table 5. *Synthesis and degradation of S-adenosylmethionine in Rps. spheroides* 8253 and 2/33

The organisms were grown aerobically in malate-glutamate medium (M-G medium) supplemented with L-methionine (0.1 M) as shown. Extracts (3 mg. of protein) were incubated for 2 hr. at 37° in mixture M3 (Expt. 1) and in mixture M4 (Expt. 2).

Strain of <i>Rps. spheroides</i>	Supplement to M-G medium	SAM (μmoles)	
		Synthesized (Expt. 1)	Degraded (Expt. 2)
8253	None	34	15
8253	Methionine	15	—
2/33	Methionine	16	13

Fig. 2. Replacement of SAM for the synthesis of methionine from 5-CH₃-H₄PteGlu by extracts of *Rps. spheroides* strain 8253. Extracts (1.4 mg. of protein) were incubated at 37° in mixture M2 plus reducing system [H], with SAM (20 μM) (○) or ATP (20 mM) + MgSO₄ (5 mM) + DL-methionine (2 mM) (●).

difference in the rate of synthesis is as expected from the high K_m requiring an initial accumulation of SAM.

DISCUSSION

Unlike *E. coli*, the photosynthetic bacteria are capable of producing vitamin B₁₂. In fact, when *Rps. spheroides* is grown in the presence of Co²⁺ it produces quantities of the vitamin comparable with those produced by organisms that are used as commercial producers of the vitamin (Volcani, Toohey & Barker, 1961).

Because of competence in the production of vitamin B₁₂ it might be expected that methyl transfer from methylated folate to homocysteine in the biosynthesis of methionine in *Rps. spheroides* might differ from that in *E. coli*, which can accomplish the transfer independent of vitamin B₁₂. When extracts of *Rps. spheroides* (wild-type) were incubated with extracts of *E. coli* methionine mutants to test for the presence in *Rps. spheroides* of the enzymes that were absent from the *E. coli* mutants, it was found that *Rps. spheroides* activates extracts of organisms lacking N⁵N¹⁰-methylene-tetrahydrofolate reductase; apparently it contains this enzyme, which is necessary for the creation of the methyl group from the methylene group on tetrahydrofolate. However, extracts of *Rps. spheroides* in conjunction with extracts of *E. coli* deficient in cobalamin-independent N⁵-methyl-tetrahydrofolate - homocysteine transmethyrase

produced only insignificant amounts of methionine in the absence of SAM; wild-type *Rps. spheroides*, unlike *E. coli*, apparently does not produce the cobalamin-independent enzyme. Thus it is similar to *E. coli* in steps preceding the creation of the methyl group but dissimilar in homocysteine methylation.

Assay for the enzyme that can utilize 5-CH₃-H₄PteGlu as substrate, and requires the cofactors SAM, FADH₂ and methylcobalamin, revealed the presence of an enzyme in *Rps. spheroides* 8253 that has absolute dependence on the addition of SAM and a reducing system. The peculiar cofactor requirements for this enzyme, together with its ability to use either 5-CH₃-H₄PteGlu or 5-CH₃-H₄PteGlu₃ as substrate, strongly suggests that *Rps. spheroides* has a cobamide-containing trans-methylase similar to that of *E. coli*. However, extracts of *Rps. spheroides* do not respond to methylcobalamin *in vitro* as do those of *E. coli*; this is presumably because *Rps. spheroides* synthesizes during growth (even in the absence of added Co²⁺) sufficient cobalamin to convert all the apoenzyme into active holoenzyme. Repeated attempts to demonstrate *in vitro* a cobalamin requirement by treating the enzymic extracts so as to remove endogenous vitamin B₁₂ (by dialysis, gel filtration and charcoal extraction) were unsuccessful. As in *E. coli*, S-adenosylhomocysteine does not replace SAM, indicating that probably the role of this cofactor is the same in both organisms and is not as an intermediate carrier of the methyl group.

With strain 2/33 the results suggest that the inability to synthesize methionine is due to lack of the apoenzyme of N⁵-methyltetrahydropteroyl-monoglutamate - homocysteine transmethyrase. Nevertheless the mutant strain has methylcobalamin-homocysteine-transmethyrase activity. The presence of this enzyme has previously been taken as an indication of the potentiality to transfer methyl groups from 5-CH₃-H₄PteGlu to homocysteine via a cobalamin-dependent route (Woods, Foster & Guest, 1964).

This difficulty would be resolved if the mutant had an anomalous SAM metabolism (either increased degradation or decreased synthesis). This has been shown not to be so. It is also possible to explain the enzyme deficiency of strain 2/33 as an inability to form the prosthetic group of a cobamide-containing transmethyrase, i.e. a defect in cobalamin metabolism rather than in apoenzyme formation. But, if this were the case, it would then be difficult to reconcile the inability of exogenous methylcobalamin to activate the enzymic extracts since this form of cobalamin directly activates the apoenzyme of *E. coli* (Foster *et al.* 1964a). Further, the mutant produces the same amount of vitamin

B₁₂ (measured as cyanocobalamin) as does the parent. An alternative explanation is that the mutant strain has an altered enzyme, incapable of the overall transfer of methyl groups from 5-CH₃-H₄PteGlu to homocysteine, but retaining the capacity to transfer such groups from methylcobalamin.

The conclusion of this paper is that wild-type *Rps. spheroides* does not have alternative mechanisms for the methylation of homocysteine. It contains only one enzyme catalysing methionine synthesis from homocysteine and that enzyme is dependent on SAM and a reducing system, and is non-specific with respect to the degree of conjugation of the methylated tetrahydrofolate supplying the methyl group. This mechanism is similar in these properties to the cobalamin-dependent enzyme of the two alternative enzymic mechanisms in *E. coli* and *S. typhimurium*.

Extracts of strain 2/33 contain amounts of vitamin B₁₂ comparable with those of the wild-type, and, since the addition of the vitamin to the growth medium does not produce growth and the addition of methylcobalamin to enzymic extracts does not lead to competent homocysteine methylation, it is not blocked in vitamin B₁₂ synthesis. Therefore it is concluded that *Rps. spheroides* strain 2/33 is probably deficient in the synthesis of the apoenzyme of the vitamin B₁₂-dependent homocysteine-methylating enzyme.

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