Methionine Synthesis by Extracts of *Salmonella typhimurium*

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1. Following the genetic studies by Smith (1961) and Smith & Childs (1963) with methionine auxotrophs of *Salmonella typhimurium*, methionine formation from homocysteine has been investigated with cell-free extracts of this organism. 2. As found with *Escherichia coli* (Woods, Foster & Guest, 1964), methyl groups are formed by an N^5N^{10} -methylenetetrahydrofolate reductase. They are then transferred to homocysteine by either a simple N^5 -methyltetrahydropteroyl-triglutamate-homocysteine methyltransferase or alternatively a cobalamin-dependent N^5 -methyltetrahydrofolate-homocysteine methyltransferase. 3. *S. typhimurium* differs from *E. coli* in being able to synthesize significant amounts of cobalamin.

Escherichia coli has alternative enzymic mechanisms for the conversion of homocysteine into methionine (Woods, Foster & Guest, 1964). These mechanisms differ at a point subsequent to the creation of the methyl group as methyltetrahydrofolate (Guest, Foster & Woods, 1964a).

In one mechanism, methyl transfer to homocysteine from either N^5 -methyl- H_4PtG ‡ or N^5 -methyl- H_4PtG_3 is catalysed by a cobamide-containing enzyme (Guest, Friedman, Foster, Tejerina & Woods, 1964b); essential cofactors for this reaction are SAM and $FADH_2$ (Rosenthal & Buchanan, 1963; Foster, Dilworth & Woods, 1964a). In the alternative reaction, specifically N^5 -methyl- H_4PtG_3 is used as substrate by N^5 -methyl- H_4PtG_3 -homocysteine methyltransferase, an enzyme that apparently does not contain cobalamin (Foster, Tejerina, Guest & Woods, 1964b); this enzyme requires only Mg^{2+} ions and is not active with N^5 -methyl- H_4PtG (Guest *et al.* 1964b). Both methyltetrahydrofolates are formed from the corresponding N^5N^{10} -methylene derivatives (in turn formed from serine via serine hydroxymethyltransferase) by the action of N^5N^{10} -methylenetetrahydrofolate reductase (Guest *et al.* 1964a). These relationships are summarized in

Scheme 1. The cobalamin-dependent pathway, in contrast with the parallel cobalamin-independent pathway, can operate only when vitamin B_{12} is supplied exogenously to *E. coli*, since this organism does not synthesize significant amounts of the vitamin (Foster *et al.* 1964b; Woods *et al.* 1964). On the other hand, the apoenzyme of the cobamide enzyme can be activated by the addition of cobalamin to either the growth medium or ultrasonic extracts of the organism (Kisliuk & Woods, 1960; Guest & Woods, 1960; Takeyama, Hatch & Buchanan, 1961).

Mutation to loss of N^5 -methyl- H_4PtG_3 -homocysteine methyltransferase gives an organism requiring cobalamin or methionine for growth, whereas mutation to loss of the N^5N^{10} -methylene-tetrahydrofolate reductase produces an organism responding to methionine alone (Woods *et al.* 1964).

Mutants of *Salmonella typhimurium* LT-2 with these phenotypes are also known and have been studied from a genetic standpoint by Smith (1961) and Smith & Childs (1963). These workers obtained evidence (from experiments using nutritional techniques, syntrophism, phage transduction and gene transfer mediated by colicine-determinants) that methionine formation from homocysteine in *S. typhimurium* is probably similar to that in *E. coli*. The object of the present work was to provide evidence at the enzymic level for the mechanism of homocysteine methylation in *S. typhimurium*.

MATERIALS AND METHODS

Organisms. *E. coli* strain 121/176 is an auxotroph derived from the prototrophic strain W (A.T.C.C. no. 9637) and requires either methionine or cobalamin for growth (Davis &

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‡ Abbreviations: for members of the folic acid group, based on PtG for pteroylglutamate; H_4PtG , tetrahydropteroylmonoglutamate; H_4PtG_3 , tetrahydropteroyltriglutamate; derivatives of these compounds are indicated as, e.g., N^5 -methyl- H_4PtG (the term folate is used in this paper in reference to both simple and conjugated members of the folic acid family of vitamins); SAM, *S*-adenosylmethionine; EHC, extract of heated *E. coli*.

Scheme 1. Two pathways for the synthesis of methionine from homocysteine and serine in *E. coli*. H₄PtG₃ refers to either H₄PtG or H₄PtG₃. ---→ indicates action with N⁵-methyl-H₄PtG₃ only.

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. no. 9723) in this Laboratory by the technique of Adelberg & Meyers (1953). Strain 121/176 lacks N⁵-methyl-H₄PtG₃-homocysteine methyltransferase, and strain 3/62 lacks N⁵N¹⁰-methylene-tetrahydrofolate reductase (Foster *et al.* 1964b).

The auxotrophic strains of *S. typhimurium* LT-2 were obtained from Dr D. A. Smith. Strains E-197, E-205, E-47, E-230 and E-235 require either cobalamin or methionine for growth. Strain F-100 responds only to methionine (Smith & Childs, 1963). Strains A-15, B-23 and C-50 respond to either homocysteine or methionine, although not to cysteine. All organisms were maintained on slopes of nutrient agar (Oxo Ltd., London, E.C.4) subcultured monthly, incubated for 16 hr. at 37° and stored at 4°.

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.

Ultrasonic extracts. They 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 extracts was determined spectrophotometrically according to Layne (1957).

Extracts were treated so as to remove cofactors by gel filtration on Sephadex G-50 (Pharmacia Ltd., London, W.13) according to the method of Kisliuk (1960), and then by extraction with charcoal according to the method of Guest *et al.* (1964b).

EHC. An extract of heated *E. coli*, 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 H₄PtG₃ 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.; where the source of folic acid cofactor was EHC 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 1.5 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 [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), 55 μmoles; DL-homocysteine, 12.3 μmoles; 2-mercaptoethanol, 11.7 μmoles; MgSO₄, 2.5 μmoles. The additions made were: SAM, 1 μmole; ATP, 200 μmoles; FAD, 75 μmoles; a NADH₂-generating system including NAD (250 μmoles), ethanol (54 μmoles) and alcohol dehydrogenase (90 μg.); methylcobalamin, 5 μmoles. Enzyme preparation equivalent to 2.5 mg. of protein was added. Substrates were either (±)-N⁵[Me-¹⁴C]-methyl-H₄PtG₃, 1.1 μmoles (0.37 μC), or (±)-N⁵[Me-¹⁴C]-methyl-H₄PtG₃, 1.6 μmoles (0.80 μC), per incubation volume.

The reaction mixtures were incubated at 37° in an atmosphere of H₂ for 30–45 min. The incubated reaction mixtures were passed through columns of Dowex 1 resin (X8; Cl⁻ form; 100–200 mesh) to remove unchanged folate as described briefly by Foster *et al.* (1964a). After washing the columns twice with 1 ml. of water, samples (0.2 ml.) of the combined effluent and washings were dissolved in 7 ml. of scintillation fluid containing 0.4% (w/v) of *p*-terphenyl

and 0.07% (w/v) of 2,2'-*p*-phenylenebis-(5-phenyloxazole) in toluene-ethanol-dioxan (12:5:3, by vol.). Radioactivity was estimated at 70% efficiency with an automatic tritium-scintillation counter (type 6020; Isotope Developments Ltd., Reading, Berks.). None of the materials present caused significant quenching of [*Me*-¹⁴C]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 methyltetrahydrofolate and agreed with values obtained by occasional random microbiological assays.

Chemicals. (±)-*N*⁵[*Me*-¹⁴C]-Methyltetrahydrofolates were prepared by reduction of (±)-*N*⁵*N*¹⁰-methylene-tetrahydrofolates and purified by column chromatography as described by Guest *et al.* (1964a). Solutions in potassium phosphate buffer, pH 7.5 (50 mM), containing 2-mercapto-ethanol (7 mM), were standardized spectrophotometrically (by using ϵ 28000 M⁻¹ cm.⁻¹ at 290 mμ) and stored under H₂ at -20°. *S*-Adenosyl-L-methionine was isolated from yeast according to 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.

Assay of cobalamin. The extracts were assayed for cobalamin with *Euglena gracilis* var. *bacillaris* (after release of the vitamin by heating and papain digestion) 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, Jones & Woods, 1961); in this case release of the vitamin was obtained by heating with HCl at 100° according to Ford, Holdsworth & Kon (1955).

RESULTS

Synthesis of methionine by extracts of methionine auxotrophs. Ultrasonic extracts of *S. typhimurium* A-15, B-23 and C-36 form methionine from homocysteine and serine when EHC is provided as the source of folic acid coenzyme (Table 1). This is to be expected since these strains are able to grow when supplied with homocysteine instead of methionine. As with *E. coli* (Szulmajster & Woods, 1960; Jones, Guest & Woods, 1961) synthesis is decreased if H₄PtG is added with EHC. However, in contrast with *E. coli*, H₄PtG does not give complete inhibition and is partially effective as a cofactor without added cobalamin (Table 1). The addition of cyanocobalamin to the extracts increases the synthesis with H₄PtG to that given by EHC.

Extracts of *S. typhimurium* E-47 (which requires either vitamin B₁₂ or methionine) unexpectedly

Table 1. *Synthesis of methionine by extracts of S. typhimurium auxotrophs*

Incubation was in mixture M1. Additions (per ml. final vol.) were: EHC, 0.1 ml.; H₄PtG, 1 μmole; cyanocobalamin, 0.74 μmole.

Strain	L-Methionine formed (mμmoles/mg. of protein/hr.) with			
	EHC	EHC+ H ₄ PtG	H ₄ PtG	H ₄ PtG+ cyanocobalamin
A-15	66	49	48	66
B-23	53	37	30	50
C-36	38	27	22	36
E-47	16	20	19	19
F-100	5	—	—	—

Table 2. *Test for N⁵N¹⁰-methylene-tetrahydrofolate reductase and N⁵-methyl-H₄PtG₃-homocysteine methyltransferase in S. typhimurium auxotrophs*

Incubation was in mixture M1 with EHC as folic acid cofactor; each extract added was equivalent to 2.5 mg. of protein.

Strain	L-Methionine formed (mμmoles/mg. of total protein)		
	Alone	With <i>E. coli</i> 3/62	With <i>E. coli</i> 121/176
<i>E. coli</i> 3/62	8	—	200
<i>E. coli</i> 121/176	20	200	—
<i>S. typhimurium</i> E-197	30	130	24
<i>S. typhimurium</i> E-235	43	132	27
<i>S. typhimurium</i> E-47	100	215	58
<i>S. typhimurium</i> E-205	72	210	48
<i>S. typhimurium</i> E-230	75	210	46
<i>S. typhimurium</i> F-100	20	18	193

form methionine without the addition of cobalamin (Table 1), in contrast with results obtained by Guest *et al.* (1960) with an analogous cobalamin/methionine mutant of *E. coli* strain 121/176. Methionine formation with EHC as cofactor, or with H₄PtG, in the presence of cobalamin is, however, significantly less than that of the other strains under the same conditions. Strain F-100, the auxotroph with a growth response to methionine alone, formed only very small amounts of methionine with EHC as cofactor.

Synthesis of methionine by mixtures of cell-free extracts. *E. coli* strain 3/62, a methionine auxotroph, lacks *N*⁵*N*¹⁰-methylene-tetrahydrofolate reductase; activation by test extracts of extracts of this strain to form methionine can be used to detect the reductase, e.g. in *E. coli* 121/176 (Guest *et al.* 1964a). Extracts of the cobalamin/methionine auxotrophs

of *S. typhimurium*, the *E* strains, increased methionine synthesis by extracts of *E. coli* 3/62 (Table 2), indicating that these strains contain the reductase. *S. typhimurium* strain *F*-100 does not do so, i.e. it lacks this enzyme.

Similarly, *E. coli* 121/176, a cobalamin/methionine auxotroph, lacks N^5 -methyl- H_4 PtG₃-homocysteine methyltransferase and, if vitamin B₁₂ is absent from the test system, activation of extracts of this organism can be used as a test for this enzyme (Foster *et al.* 1964b). Extracts of the cobalamin/methionine auxotrophs of *S. typhimurium* have very little of this enzyme (Table 2), i.e. *S. typhimurium* *E* mutants are analogous in their metabolic lesion to *E. coli* 121/176. In strain *F*-100, on the other hand, the methyltransferase is present.

As would then be expected, mixtures of strains *E* and *F*, like mixtures of *E. coli* 3/62 and 121/176, formed methionine readily, e.g. extract of strain *F*-100 with *E*-235 made 160 μ moles of L-methionine/mg. of protein/hr. and 266 μ moles/mg. of protein/hr. with *E*-230.

The *S. typhimurium* cobalamin/methionine auxotroph can be divided into two genetic complementation groups (Smith & Childs, 1963). Of the strains studied, *E*-197 and *E*-235 are in one complementation group; *E*-47, *E*-205 and *E*-230 are in the other. Cell-free extracts might therefore exhibit complementation *in vitro* when incubated together. Significant increase of methionine synthesis, however, did not occur under these conditions (Table 3). On the other hand, different N^5N^{10} -methyltetrahydrofolate-reductase activities in the two groups were observed (Table 2, penultimate column); this observation remains unexplained, since it is not clear how mutation to loss of the methyltransferase might affect the reductase activity, particularly when the two genes controlling the syntheses of these enzymes are not linked (Smith, 1961). If the mutation in the

complementation group with low reductase activity were to produce a transmethylease so modified as to be enzymically inactive but still to bind strongly the small amounts of methyltetrahydrofolate formed at any one time from serine, then an inhibition of methionine formation would occur in the test for the reductase by admixture with *E. coli* 3/62. This hypothesis was tested by incubating extracts of mutants (*E*-225 and *E*-230) from each of the two genetic complementation groups in turn with mixed extracts of *E. coli* strains 121/176 and 3/62, i.e. providing both the reductase and methyltransferase from *E. coli*. However, the *S. typhimurium* extracts did not affect the methionine synthesis by the mixture of *E. coli* 3/62 and 121/176, indicating that the hypothesis is not correct.

Cobalamin and the methylation of homocysteine.

(a) Organisms grown without added vitamin B₁₂. In *E. coli* methyl transfer from N^5 -methyl- H_4 PtG to homocysteine is catalysed by a cobamide-containing enzyme, which can be formed *in vitro* from methylcobalamin and an apoenzyme present in organisms grown without the vitamin. The action of this enzyme requires SAM as cofactor (Foster *et al.* 1964a).

Extracts of *S. typhimurium* also catalysed this methyl transfer (Table 4). However, in the absence of methylcobalamin, there was still considerable synthesis of methionine from N^5 -methyl- H_4 PtG, even with the cobalamin auxotrophs. This contrasted sharply with *E. coli*, where activity depends absolutely on the addition of a cobalamin. This synthesis, in the absence of methylcobalamin, was dependent on the addition of SAM and a reducing system. These requirements suggest strongly that the activity observed represented an endogenous

Table 3. Complementation test *in vitro* with cobalamin auxotrophs of *S. typhimurium*

Incubation was in mixture M1 with EHC as folic acid cofactor; each extract added was equivalent to 2.5 mg. of protein.

Strain	L-Methionine formed (μ moles/mg. of total protein) with strain				
	<i>E</i> -197	<i>E</i> -235	<i>E</i> -47	<i>E</i> -205	<i>E</i> -230
<i>E</i> -197	30	36	62	44	58
<i>E</i> -235	36	43	66	60	60
<i>E</i> -47	62	66	100	86	80
<i>E</i> -205	44	60	86	72	74
<i>E</i> -230	58	60	80	74	75

Table 4. N^5 -Methyl- H_4 PtG-homocysteine methyltransferase in extracts of *S. typhimurium*

Extracts were from organisms grown with methionine as supplement and treated to remove cofactors. Incubation was for 45 min. in mixture M2 with $N^5[Me-^{14}C]$ -methyl- H_4 PtG. 'NADH₂' refers to the NADH₂-generating system described in the Materials and Methods section.

Addition	L-Methionine formed (μ moles/mg. of protein/hr.) with extracts of strain			
	<i>LT</i> -2	<i>E</i> -230	<i>E</i> -235	<i>F</i> -100
None	21	3	5	19
SAM	—	70	62	—
FAD, 'NADH ₂ '	48	30	45	—
SAM, FAD, 'NADH ₂ '	180	130	130	184
SAM, FAD, 'NADH ₂ ', methylcobalamin	220	220	190	220
FAD, 'NADH ₂ ', methylcobalamin	62	51	45	40

Table 5. *N*⁵-Methyl-*H*₄PtG₃-homocysteine methyltransferase in extracts of cobalamin/methionine auxotrophs of *S. typhimurium* grown with vitamin B₁₂

Incubation was for 30 min. in mixture M2 with *N*⁵-[Me-¹⁴C]-methyl-*H*₄PtG₃. 'NADH₂' refers to the NADH₂-generating system described in the Materials and Methods section.

Addition	L-Methionine formed (mμmoles/mg. of protein/hr.) with extract of strain	
	<i>E</i> -235	<i>E</i> -230
None	0	0
SAM	4	10
FAD, 'NADH ₂ '	6	12
SAM, FAD, 'NADH ₂ '	280	293
ATP, FAD, 'NADH ₂ '	28	31
ATP, Mg ²⁺ , FAD, 'NADH ₂ '	280	285
SAM, FAD, 'NADH ₂ ', methylcobalamin	278	288

cobamide holoenzyme (rather than a new enzyme similar to *N*⁵-methyl-*H*₄PtG₃-homocysteine methyltransferase of *E. coli* but without the latter's folate specificity) since the cobamide enzyme of *E. coli*, when formed *in vivo*, requires FADH₂ as well as SAM for activity *in vitro*.

The results of Table 4 show that some cobamide apoenzyme is also present, since the further addition of methylcobalamin increases the methionine formed in the presence of SAM and the reducing system. Even in the presence of methylcobalamin, the omission of SAM still decreases methionine formation to the same extent.

(b) Organisms grown with added vitamin B₁₂. When *S. typhimurium* *E*-235 and *E*-230 were grown with vitamin B₁₂ instead of methionine as supplement, there was no longer any effect of methylcobalamin *in vitro*, but there was now an absolute requirement for a reducing system (Table 5). Similar results were obtained with *E. coli*, where the cobamide enzyme, formed *in vivo*, requires after extraction reactivation by FADH₂ before participation in methyl transfer (Foster *et al.* 1964a). As with extracts from organisms grown without vitamin B₁₂, SAM was required and could be replaced by ATP and Mg²⁺.

Occurrence of cobalamin in S. typhimurium. The results above with organisms grown without vitamin B₁₂ suggested that, in contrast with *E. coli*, *S. typhimurium* has an endogenous vitamin B₁₂-dependent homocysteine methylation. This hypothesis was tested by measuring the vitamin B₁₂ content of thick suspensions of *S. typhimurium* grown on unsupplemented medium (wild-type) or with methionine supplement (auxotrophs). The

Table 6. Methionine formation from *N*⁵-methyl-*H*₄PtG₃

Extracts were from organisms grown with methionine as supplement and treated to remove cofactors. Incubation was for 45 min. in mixture M2 with *N*⁵[Me-¹⁴C]-methyl-*H*₄PtG₃. 'NADH₂' refers to the NADH₂-generating system described in the Materials and Methods section.

Addition	L-Methionine formed (mμmoles/mg. of protein/hr.) with extracts of strain	
	<i>E</i> -235	<i>F</i> -100
None	8	300
SAM	20	—
FAD, 'NADH ₂ '	10	—
SAM, FAD, 'NADH ₂ '	80	—
SAM, FAD, 'NADH ₂ ', methylcobalamin	250	380

results (expressed as μg. of cyanocobalamin/g. dry wt.) ranged from 300 to 1000 (measured with *Euglena*) or from 700 to 1100 (measured with *E. coli* 113/3). This is considerably more than that reported for *E. coli* (3 μg./g. dry wt.) by Foster *et al.* (1964b). These authors calculated that the *E. coli* value corresponded to less than 0.5 mol. of vitamin B₁₂/cell. Correspondingly, *S. typhimurium* has then about 50–200 mol. of vitamin B₁₂/organism, i.e. contains a significant amount even when growing in a medium without cobalamin. It should be noted that the same quantity of cobalamin was found in the cobalamin auxotrophs as in the wild type.

Cobalamin-independent methylation of homocysteine. The studies with mixtures of cell-free extracts showed that the methionine auxotroph, strain *F*-100, contained *N*⁵-methyl-*H*₄PtG₃-homocysteine methyltransferase. This was confirmed by direct assay with the defined substrate (Table 6). In contrast, extracts of the cobalamin auxotroph, *E*-235, did not catalyse methyl transfer from *N*⁵-methyl-*H*₄PtG₃ to homocysteine unless supplemented with SAM, methylcobalamin and a reducing system, i.e. unless the cobalamin-dependent pathway was brought into operation. The addition of these cofactors to extract of strain *F*-100 was not necessary for methyl transfer from *N*⁵-methyl-*H*₄PtG₃ compared with transfer from *N*⁵-methyl-*H*₄PtG (see Table 4), but did enhance methionine formation to some extent.

DISCUSSION

Substantial methionine synthesis, comparable with that of *E. coli* (Foster *et al.* 1964b) and *Aerobacter aerogenes* (Morningstar & Kisliuk, 1965), was found to occur from homocysteine and serine with

ultrasonic extracts of *S. typhimurium*, except where these were derived from auxotrophs not responding to homocysteine for growth. The suggestion from the nutritional results (Smith, 1961) that enzymes similar to those in *E. coli* are present in *S. typhimurium* was borne out at the enzymic level. For example, H_4PtG is inhibitory to the action of EHC as folic cofactor but becomes, itself, a fully competent cofactor when vitamin B_{12} is added *in vitro* to vitamin-deficient extracts. Again, the formation of methyl groups appears to be via a methylenetetrahydrofolate reductase as in *E. coli*, since an extract of *S. typhimurium* will activate an extract of *E. coli* that specifically lacks this enzyme; the reductase was absent from strain *F*-100, accounting for the methionine requirement of this strain.

The transfer of methyl groups to homocysteine occurs by two routes, as in *E. coli*. There is, for example in strain *F*-100, an enzyme that transfers methyl groups to homocysteine from N^5 -methyl- H_4PtG_3 but not from N^5 -methyl- H_4PtG . This did not require the addition of cofactors such as SAM and methylcobalamin, as did transfer from N^5 -methyl- H_4PtG with the same strain. This apparently simple cobalamin-independent transmethylation was specifically lacking from the cobalamin/methionine auxotrophs. The division of these auxotrophs into two distinct complementation groups could not be interpreted as evidence for two separate enzymic steps in the methyl transfer reaction because no synergism was found between extracts from the two groups, although the production of fully competent extracts from the methionine auxotroph shows that any two such enzymes would be stable to extraction and assay. Further, no evidence was found for complementation *in vitro* arising from the recombination of enzyme sub-units.

In the cobalamin auxotrophs, there was a cobalamin-dependent methyl transfer, which was active with either N^5 -methyl- H_4PtG or N^5 -methyl- H_4PtG_3 and required SAM (and a source of reducing power if the cobalamin originated *in vitro*). These mutants were originally puzzling because their extracts were able to form methionine to the extent of 40% of that given by a competent strain with the source of natural folic acid as cofactor (Table 1), and yet these organisms showed no signs of 'leaky' growth in the absence of a methionine supplement (Smith, 1961). The results with the cofactor-dependent methyl transfer and the measurements of endogenous vitamin B_{12} indicate that *S. typhimurium*, and the cobalamin auxotroph in particular, differs from *E. coli* in being able to synthesize cobalamin to a limited, though significant, extent. Therefore this organism has a potential cobalamin-dependent methyl transfer that would operate even when the alternative

vitamin-independent mechanism has been lost by mutation. Nevertheless, the vitamin synthesized is patently insufficient to satisfy the growth requirement. Calculations (Cauthen, 1965) have shown that growth of the cobalamin auxotrophs of *E. coli* (which does not synthesize vitamin B_{12}) ceases when the vitamin content of the cells falls to the levels found as endogenous material in *S. typhimurium*. The significance of the limited ability of *S. typhimurium* to synthesize cobalamin is not clear.

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