Transfer of the Methyl Group from N^5 -Methyltetrahydrofolates to Homocysteine in *Escherichia coli*

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(Received 23 January 1964)

Evidence was given in the two preceding papers that strains of *Escherichia coli* possess alternative enzymic mechanisms for the conversion of homocysteine into methionine, and that these mechanisms differ at a point subsequent to the creation of the methyl group (Foster, Tejerina, Guest & Woods, 1964*b*; Guest, Foster & Woods, 1964*a*). One mechanism requires, specifically, N^5 -methyltetrahydropteroyltriglutamate* as methyl donor and is catalysed by an enzyme (*B*) which does not contain cobalamin. In the other, either N^5 -methyl- H_4 PtG or N^5 -methyl- H_4 PtG₃ serves as a methyl donor but a cobamide-containing enzyme (Foster, Jones & Woods, 1961) is an essential component of the enzyme system.

The work now described is a detailed study of the cofactor and other requirements associated with the two mechanisms. The term folate is used in general reference to both mono- and tri-glutamate derivatives. A brief report of some of the observations has been given by Guest, Friedman & Foster (1962).

MATERIALS AND METHODS

Sources of enzymes

Organisms. The methods used for the maintenance and bulk growth of the three auxotrophic strains of *E. coli* used were as described by Guest, Helleiner, Cross & Woods (1960), as also was the preparation from them of ultrasonic extracts. *E. coli* 121/176 requires for growth either methionine or cobalamin, whereas strain 3/62 responds only to methionine; their content of relevant enzymes under differing growth conditions is summarized in Table 1 of Guest *et al.* (1964*a*). *E. coli* PA15, which requires for growth either serine or glycine, is fully competent to synthesize methionine from homocysteine and serine. The basal growth medium was normally supplemented with glycine or glycine plus cobalamin (strain PA15), methionine or methionine plus cobalamin (strain 3/62), and methionine or cobalamin (strain 121/176); the concentrations used were: glycine, 10 mM; DL-methionine, 0.4 mM; cobalamin, 18.5 μ M.

Enzyme preparations. An ultrasonic extract of *E. coli* 3/62 (grown with methionine) was used directly as source of

enzyme *B* in some experiments; it has the advantage of being free from methylenetetrahydrofolate reductase (enzyme *A*) which could oxidize the N^5 -methyltetrahydrofolate used as methyl donor for the methylation of homocysteine. Preparations purified 7–12-fold were prepared by the method of Foster *et al.* (1964*b*).

An extract of *E. coli* 121/176 was sometimes used as direct source of the cobamide-containing enzyme free from enzyme *B*. Purified cobamide-containing enzyme was prepared from an aqueous extract of acetone-dried *E. coli* PA15 (grown with cobalamin) by the modified procedure of Foster *et al.* (1964*b*); it was finally precipitated by dialysis at 2° for 6 hr. against 20 vol. of $(NH_4)_2SO_4$ solution (90% saturated, adjusted to pH 7.8 with NH_3). After centrifuging for 5 min. at 25 000*g*, the bulk of the supernatant fluid was removed and the sediment stored at 2° in the remaining fluid. This preparation had a half-life of 3 weeks and the specific activity, determined according to the method of Foster *et al.* (1961), indicated a purification of 140–180-fold with an overall recovery of 5%. Before use samples of the suspension were centrifuged; the pellet was dissolved in 50 mM-potassium phosphate, pH 8.7, and the solution dialysed for 2 hr. against the same buffer.

Extracts in which both the cobamide-containing enzyme and enzyme *B* were present were obtained from either *E. coli* 3/62 or PA15 after growth with cobalamin plus either methionine or glycine respectively.

The enzyme (or enzymes) designated protein fraction *Y* were present in all three strains of *E. coli* irrespective of whether or not they had been grown with cobalamin. Material enriched 2–3-fold was obtained from *E. coli* PA15 during the purification of the cobamide-containing enzyme. The protein eluted from Celite 545 by 75 mM- and 120 mM-potassium phosphate, pH 7.8, was combined and precipitated by dialysis against $(NH_4)_2SO_4$ solution (90% saturated); it was stored and prepared for use as described for the purified cobamide-containing enzyme.

Removal of cofactors and metal ions. Ultrasonic extracts used directly as sources of enzymes were dialysed against 50 mM-phosphate buffer, pH 7.8, and treated with Dowex 1 resin (X8; Cl^- form; 100–200 mesh) as described by Kisliuk & Woods (1960) immediately before use. Both crude and purified preparations of enzyme *B* were also treated with charcoal (acid-washed and ground Nuchar C; West Virginia Pulp and Paper Co., New York, U.S.A.): the amount of charcoal used/mg. of protein was 0.4 and 3.0 mg. respectively for the crude and purified material. After stirring for 20 min. at 0° the charcoal was removed by twice centrifuging at 25 000*g* for 5 min.

Four methods were used to remove metal ions from purified preparations of enzyme *B*; they were carried out in triethanolamine buffer, pH 7.8. The preparation was treated for 2 hr. at 0° with either (1) potassium cyanide (10 mM) or

* Abbreviations for members of the folic acid group, based on PtG for pteroyltriglutamate; H_4 PtG, tetrahydropteroylmonoglutamate; H_4 PtG₃, tetrahydropteroyltriglutamate; derivatives of these compounds are indicated as, e.g., N^5 -methyl- H_4 PtG.

(2) EDTA (disodium salt) (25 mM) and the reagent removed by dialysis against two changes of 20 vol. of 100 mM-triethanolamine buffer, pH 7.8. (3) The preparation (5 ml.; 1 mg. of protein/ml.) was applied to a column (12 cm. $\times$ 1.5 cm.²) of Sephadex G-25 (Pharmacia, Uppsala, Sweden) gel in 100 mM-triethanolamine buffer, pH 7.8. (4) The preparation was shaken gently for 2 hr. at 0° with Chelex-100 (Dowex A 1) chelating resin (K⁺ form; 50–100 mesh).

Test for methionine formation

Cobalamin-independent mechanism. The basal reaction mixture, R₁, contained (in final vol. 1 ml.): DL-homocysteine, 8 μ moles; ($\pm$)-N⁵-methyl-H₄PtG₃, 1.5 μ moles; MgSO₄, 5 μ moles; potassium phosphate buffer, pH 7.8, 100 μ moles. Supplements used as stated in certain experiments were: ATP, 5 μ moles; an enzymic reducing system (FAD, 0.1 μ -mole; NAD, 1 μ mole; ethanol, 40 μ moles; crystallized alcohol dehydrogenase, 150 μ g.). In tests of the metal ion requirements of the enzyme the phosphate buffer was replaced by 100 μ moles of triethanolamine buffer, pH 7.8. The amount of enzyme B added (0.05–1.0 mg. of protein) depended on the purity of the preparation used and serum albumin was included to bring the final protein concentration to 2 mg./ml. Incubation was at 37° in air for 20–30 min. and the reaction was stopped by heating the mixture at 100° for 3 min. Precipitated protein was removed by centrifuging and methionine estimated in the supernatant fluid by the microbiological assay of Gibson & Woods (1960). The value obtained (15–20 μ m-moles) in each test for a control containing heated enzyme has been deducted from the experimental values recorded.

Cobalamin-dependent mechanism. The basal reaction mixture, R₂, contained (in final vol. 1.6 ml.): DL-homocysteine, 10 μ moles; ($\pm$)-N⁵-methyl-H₄PtG, 2.5 μ moles, or ($\pm$)-N⁵-methyl-H₄PtG₃, 1.5 μ moles; ATP, 10 μ moles; MgSO₄, 10 μ moles; FAD, 0.2 μ mole; NAD, 1 μ mole; ethanol, 40 μ moles; alcohol dehydrogenase, 150 μ g. The source of enzyme was either a mixture of purified cobamide-containing enzyme (20–40 μ g. of protein) and protein fraction Y (0.4–1.0 mg. of protein) to which was added 1 mg. of bovine serum albumin, or an unpurified (with respect to protein) extract of organisms grown with cobalamin (5 mg. of protein). Incubation was for 1.5–4.0 hr. at 37° in an atmosphere of H₂. The reaction was terminated and methionine assayed as described above; the value for a control with heated enzyme was about 10 μ m-moles.

Chemicals

Folic acid derivatives. ($\pm$)-N⁵-Methyltetrahydrofolates were prepared and stored as described by Guest *et al.* (1964a) for the products of the chemical reduction of the corresponding N⁵N¹⁰-methylene derivatives. Solutions in potassium phosphate buffer, pH 7.8, were standardized spectrophotometrically at 290 m μ assuming an arbitrary molar extinction coefficient of 25 000 cm.²/mole. With N⁵-methyl-H₄PtG the product was used directly; the N⁵-methyl-H₄PtG₃, which had been prepared from the less pure sample (II) of PtG₃, was purified by chromatography on triethylaminoethylcellulose (TEAE-cellulose).

Other materials. S-Adenosyl-L-homocysteine was a gift from Dr W. Sakami and S-adenosyl-L-methionine iodide was obtained from the California Corp. for Biochemical Research, Los Angeles, U.S.A. The sources of other special

chemicals and enzymes were as given by Guest *et al.* (1964a). Potassium phosphate buffers were prepared from KH₂PO₄ and K₂HPO₄. Triethanolamine buffer was made by adjusting a solution of recrystallized triethanolamine hydrochloride to pH 7.8 with KOH.

RESULTS

Cobalamin-independent methyl transfer

It was shown in the two preceding papers (Foster *et al.* 1964b; Guest *et al.* 1964a) that extracts of *E. coli* containing enzyme B, but not the cobamide-containing enzyme, catalysed the transfer of the methyl group from N⁵-methyl-H₄PtG₃ to homocysteine. Further exploratory experiments with preparations of enzyme B treated to remove cofactors, but not purified with respect to protein, established optimum conditions for the study of this reaction and also revealed that neither ATP nor a reducing environment was apparently required. The rate of methionine formation (about 400 μ m-moles/mg. of protein/hr.) was almost four times that obtained under optimum conditions for the overall enzymic conversion of serine plus homocysteine into methionine. Investigations were thereafter carried out with a preparation of enzyme B which had also been purified about tenfold with respect to protein content; this is the highest degree of purification so far achieved (Foster *et al.* 1964b).

Kinetics. The amount of methionine formed increased linearly with time up to about 30 min. (Fig. 1) and was proportional to enzyme concentration up to about 150 μ g. of protein/ml. (Table 1).

Fig. 1. Time-course of the methylation of homocysteine by purified enzyme B of the cobalamin-independent reaction. Reaction mixture R₁ was incubated for the time stated with 150 μ g. of protein of a preparation of enzyme B that had been purified tenfold.

The deviation from linearity beyond these points was probably due to the concentration of N^5 -methyl- H_4PtG_3 having fallen below the limiting value; it was not possible, with the limited supplies

Table 1. *Effect of enzyme concentration on methionine formation by the cobalamin-independent reaction*

The preparation of enzyme *B* (purified tenfold) was incubated for 20 min. in reaction mixture R_1 .

Enzyme <i>B</i> added (μ g. of protein)	L-Methionine formed (μ m-moles)
75	77
150	140
225	185
450	245

Table 2. *Substrate specificity and cofactor requirements of the cobalamin-independent reaction*

Reaction mixture R_1 was modified as indicated and incubated for 30 min. with purified enzyme *B* (140 μ g. of protein).

Substrate (1.5 μ moles)	Modifications to reaction mixture	L-Methionine formed (μ m-moles)
N^5 -Methyl- H_4PtG_3	None	190
	MgSO ₄ omitted	132
	ATP added	198
	'NADH ₂ '* added	202
	ATP and 'NADH ₂ '* added	195
	ATP and 'NADH ₂ '* added†	197
N^5 -Methyl- H_4PtG	None	5
	ATP, 'NADH ₂ '* and FAD added†	9

* 'NADH₂' refers to the NADH₂-generating system comprising NAD, ethanol and alcohol dehydrogenase (see the Materials and Methods section).

† Incubated anaerobically.

Table 3. *Effect of magnesium ions on the cobalamin-independent reaction*

A purified preparation of enzyme *B* was further treated as indicated (for details see the Materials and Methods section) and incubated (75 μ g. of protein) for 30 min. with and without MgSO₄ (5 mM) in reaction mixture R_1 modified by omitting MgSO₄ and substituting triethanolamine buffer for phosphate.

Buffer	Treatment of the enzyme preparation	L-Methionine formed (μ m-moles)	
		Mg ²⁺ ions absent	Mg ²⁺ ions present
100 mM-Phosphate buffer, pH 7.8	None	135	135
100 mM-Triethanolamine buffer, pH 7.8	None	135	135
	Cyanide	30	150
	EDTA	35	150
	Sephadex G-25	40	140
	Chelex-100	25	140

available, to use this substrate at a higher initial concentration.

The optimum pH was not sharp; activity was maximal at pH 8.1 but fell only to 70 and 88 % of the maximum at pH 6.0 and 8.7 respectively.

Substrate specificity. Enzyme *B* was active only with N^5 -methyl- H_4PtG_3 ; the monoglutamate analogue was not used (Table 2), even on the further addition to the reaction mixture of ATP and a reducing system, i.e. cofactors required for the use of this substrate by the cobamide-containing enzyme. Other potential methyl donors which proved to be inactive were choline, betaine and creatine, but just detectable amounts of methionine were found chromatographically with *S*-methylcysteine.

Cysteine did not replace homocysteine as acceptor of the methyl group of N^5 -methyl- H_4PtG_3 , nor was the reaction with homocysteine as acceptor inhibited by cysteine.

Cofactor requirements. The use of the purified protein fraction of the enzyme *B* preparation did not reveal any accessory requirement other than for Mg²⁺ ions, the omission of which decreased methionine formation by about 30 % (Table 2). The addition of ATP, FAD and a NADH₂-generating system did not significantly increase methionine formation (Table 2). This was also the case with an aqueous extract of heated *E. coli* (Guest *et al.* 1960) which was tested as a general source of cofactors natural to this organism.

Requirement for Mg²⁺ ions. This was investigated further by so treating the enzyme preparation as to remove or form complexes with any metal ions present. Treatment with Sephadex G-25 gel, Chelex-100 resin, EDTA and cyanide all resulted in a 70–80 % loss of activity that was completely restored by the addition of Mg²⁺ ions (Table 3). It had been found previously that cobalamin-independent methyl transfer was more sensitive to cyanide than the mechanism dependent on cobalamin (Foster *et al.* 1964b); this may therefore have been due to the formation of complexes of the metal ion required by enzyme *B*.

At low concentrations Mn²⁺ ions were more effective than Mg²⁺ ions, but concentrations of Mn²⁺ ions greater than 1 mM were inhibitory and the highest levels of methionine formation were obtained with Mg²⁺ ions (5 mM) (Fig. 2). Other bivalent metal ions tested at 5 mM were only weakly active (Fe²⁺, Ca²⁺ and Zn²⁺ ions) or inactive (Co²⁺ ions); no steps were taken to purify the salts used.

Adenosine triphosphate. There was no requirement for ATP under the present test conditions with defined substrates and purified enzyme (Table 2). In earlier experiments a 2–3-fold stimulation by ATP had been found when cobalamin-

independent methylation of homocysteine was studied in more complex systems with serine as C_1 donor and H_4PtG as cofactor or with N^5N^{10} -methylene- H_4PtG_3 as direct C_1 donor (Guest, 1960; Foster *et al.* 1964*b*). Further investigation of the first of these systems, with a mixture of extracts of strains 121/176 and 3/62 as enzyme source, has shown that the ATP requirement depends on the nature of the reducing system supplied and that is

Fig. 2. Effect of Mg^{2+} and Mn^{2+} ions on the activity of the purified enzyme of the cobalamin-independent reaction. Enzyme *B* (purified tenfold) was treated consecutively with Sephadex G-25 and Chelex-100; 75 μ g. (protein) was incubated in reaction mixture R_1 containing 100 mM-triethanolamine buffer, pH 7.8, instead of phosphate and bivalent metal ion at the concentrations indicated. ●, Mg^{2+} ions; ○, Mn^{2+} ions.

Table 4. Assay of the enzyme of the cobalamin-independent reaction by independent methods

The fractions containing enzyme *B* were obtained from *E. coli* 3/62 as described by Foster *et al.* (1964*b*) and were assayed (a) as described in that paper with extracts of *E. coli* 121/176 and serine as C_1 donor and (b) by the method described in the present paper with N^5 -methyl- H_4PtG_3 as direct methyl donor in reaction mixture R_1 .

Fraction	L-Methionine formed (μ moles/mg. of enzyme <i>B</i> protein/hr.)		(a):(b) ratio
	Method (a)	Method (b)	
Crude extract	0.64	0.38	1.70
Protamine supernatant	1.56	0.90	1.75
DEAE-cellulose eluent	2.75	1.83	1.50
Dialysed eluent	3.45	1.98	1.75
Alumina C_{γ} -gel supernatant (first treatment)	3.85	2.19	1.75
Alumina C_{γ} -gel eluent (after second treatment)	7.50	4.86	1.55
DEAE-cellulose concentrate	7.90	4.48	1.75

necessary for the reduction of the C_1 donor system from hydroxymethyl to methyl level. ATP was not required if FAD were present together with a $NADH_2$ -generating system comprising NAD plus either ethanol and alcohol dehydrogenase or hexose diphosphate plus endogenous enzymes. It is concluded that ATP was required only for the phosphorylation of glucose or the conversion of FMN into FAD or both; in the earlier experiments either glucose had been used as source of reducing power or FMN as source of added flavin.

Phosphate. Replacement of phosphate buffer by triethanolamine buffer of the same pH did not diminish the yield of methionine (Table 3); the enzyme preparation and other reagents had also been prepared in the latter buffer. There is therefore no major requirement for phosphate ion in the reaction.

Alternative assay. Evidence was sought bearing on the question of whether enzyme *B* has only one enzymic component by measuring its apparent activity, by two different methods, at all stages of its isolation. One method was that employed throughout this section, i.e. the specific catalysis of the transfer of the methyl group from N^5 -methyl- H_4PtG_3 to homocysteine. The other was an assessment of its ability to complete the enzymic constitution of extracts of *E. coli* 121/176 (which lack enzyme *B*) and thus bring about the overall formation of methionine from homocysteine and serine by such extracts (Foster *et al.* 1964*b*). The ratio of the specific activities in each assay was virtually constant throughout the purification (Table 4).

Distribution. The relative activities of the strains of *E. coli* used in this work in catalysing the formation of methionine from homocysteine and N^5 -methyl- H_4PtG_3 were compared with extracts unpurified with respect to protein. The values obtained were (μ m-moles of L-methionine/mg. of protein/hr.): strain 3/62, 330; strain PA 15, 180; strain 121/176, 0.

Cobalamin-dependent methyl transfer

Further investigation of this mechanism, in which N^5 -methyl- H_4PtG as well as N^5 -methyl- H_4PtG_3 is an effective substrate, was carried out with a preparation of cobamide-containing enzyme (derived from *E. coli* PA 15 grown with cobalamin) purified about 160-fold with respect to protein. In view of its greater availability N^5 -methyl- H_4PtG was used for most of the work. During purification, the assay of the cobamide-containing enzyme was in terms of its ability to promote methionine formation from homocysteine and serine in the presence of unpurified preparations of *E. coli* PA 15 specifically devoid of cobamide-containing enzyme (Foster *et al.* 1961). Previous studies with N^5 -methyl- H_4PtG as direct methyl donor were with an

unpurified preparation of the cobamide-containing enzyme (Guest *et al.* 1964*a*). It was not known therefore whether other enzymes, possibly present in the bacterial preparations used, were required in addition to the cobamide-containing enzyme.

Need for accessory enzyme(s). A batch of purified cobamide-containing enzyme was prepared by the modified method of Foster *et al.* (1964*b*) and material at successive steps in the fractionation tested for its ability to catalyse transmethylation from N^5 -methyl- H_4 PtG to homocysteine (Table 5). The supernatant fluid after treatment with protamine sulphate (fivefold purification) was fully active, but the final material obtained after chromatography on Celite 545 (150-fold purification) was almost inactive. Activity was restored by the addition of other fractions from the Celite column, those eluted by 75 mm- and 120 mm-potassium phosphate buffer, pH 7.8, being responsible for the greater part of the effect. These fractions were pooled and designated protein fraction Y, which had a specific activity 1.5–3 times that of the original extract; its essential requirement, in addition to cobamide-containing enzyme, for methionine formation is shown in Table 6.

Kinetics. The rate of formation of methionine from homocysteine and N^5 -methyl- H_4 PtG was directly proportional to the amount of cobamide-containing enzyme with a constant amount of protein fraction Y, and directly proportional to the amount of the latter when the amount of cobamide-containing enzyme was held constant. However, even with both enzyme sources present at optimum concentrations there was always a lag, varying erratically from 30 to 90 min., before methionine formation commenced; thereafter it was directly proportional to time for about 4 hr. It was not possible in these circumstances to make a quantitative study of other aspects of the kinetics of the reaction.

Cofactors. Use of the purified sources of both cobamide-containing enzyme and protein fraction Y permitted a clear-cut demonstration of the cofactor requirements of the reaction. The single omission from the reaction mixture of ATP, Mg^{2+} ions, FAD or the $NADH_2$ -generating system decreased methionine formation to about 10 % or less (Table 6), allowing for the controls without substrate. FMN had only about one-fifth of the activity of the same concentration of FAD and was inhibitory at higher concentrations. ATP was replaced by ADP to only a limited extent, and AMP and adenosine were inactive (Table 7).

Substrate specificity. Similar results to those of Table 6 were obtained when N^5 -methyl- H_4 PtG was replaced as substrate by the corresponding tri-glutamate derivative, although considerably less methionine was formed, probably owing to the use

Table 5. Requirement of the purified cobamide-containing-enzyme system for accessory enzyme(s)

The fractions possessing the cobamide-containing enzyme were obtained from *E. coli* PA 15 as described by Foster *et al.* (1961) and were incubated (6 units of enzyme in each case) in reaction mixture R_2 . The extract (ultrasonic) of *E. coli* 3/62 after growth with methionine was dialysed, but not otherwise purified.

Source of cobamide-containing enzyme	Specific activity (units/mg. of protein)	L-Methionine formed (μ m-moles)
Crude extract	1.2	410
pH 5.0 supernatant	3.6	390
Protamine sulphate supernatant	6.0	350
Celite 545 eluent	180.0	35
Extract of <i>E. coli</i> 3/62	—	5
Celite 545 eluent + extract of <i>E. coli</i> 3/62	—	400

Table 6. Components of the cobalamin-dependent system for the methylation of homocysteine

Purified cobamide-containing enzyme (5 μ g. of protein) plus protein fraction Y (0.6 mg. of protein) were incubated for 4 hr. in reaction mixture R_2 . Enzymes, substrates and cofactors were omitted as indicated.

Component omitted	L-Methionine formed (μ m-moles)
None	615
FAD	85
*NADH_2	25
ATP	75
Mg^{2+} ions	80
Homocysteine	25
N^5 -Methyl- H_4 PtG	15
Cobamide-containing enzyme	25
Protein fraction Y	30

* *NADH_2 refers to the $NADH_2$ -generating system comprising NAD, ethanol and alcohol dehydrogenase.

Table 7. Effect of adenosine triphosphate and homocysteine on the cobamide-containing-enzyme system

The general test conditions were as for Table 6 with the modifications to reaction mixture R_2 indicated. Supplements used were ADP (5 μ moles), AMP (5 μ moles), adenosine (5 μ moles), and *S*-adenosylhomocysteine (4 μ moles) plus 2-mercaptoethanol (20 μ moles).

Modifications to the reaction mixture		L-Methionine formed (μ m-moles)
Omissions	Additions	
None	None	615
ATP	None	75
ATP	ADP	125
ATP	AMP	70
ATP	Adenosine	50
Homocysteine	None	25
Homocysteine and ATP	<i>S</i> -Adenosylhomocysteine	27
Homocysteine	<i>S</i> -Adenosylhomocysteine	88

of sub-optimum concentrations of the triglutamate, which was in short supply. The requirement for ATP and the reducing system made it clear, however, that the reaction then studied could not have been due to the presence of any enzyme *B* which, as shown above, does not require these cofactors.

S-Adenosylhomocysteine replaced homocysteine as methyl acceptor only to a very limited extent that was still dependent on the presence of ATP (Table 7). Further, *S*-adenosylmethionine, the presumptive product of methylation, did not yield methionine when incubated, either alone or in the presence of homocysteine, with unpurified bacterial extracts with or without the cobamide-containing enzyme; these extracts were derived from *E. coli* 3/62 grown with or without cobalamin respectively.

Substitution of cysteine for homocysteine in the normal reaction mixture did not yield any *S*-methylcysteine detectable either by chromatography on paper or by radioautography with N^5 -[^{14}C]methyl- H_4 PtG as methyl donor.

Properties of protein fraction Y. The activity of this material is presumed to be enzymic in nature since it is non-diffusible and destroyed by heating at 70° for 6 min.; it is, however, relatively thermostable since it is not affected by a temperature of 60° for 6 min. It was not replaced by an aqueous extract of heated whole organisms.

An unpurified extract of *E. coli* 3/62, grown with methionine and devoid of the cobamide-containing enzyme, was also able to activate the purified preparation of cobamide-containing enzyme (Table 5). It is clear therefore that the production during

growth of the enzyme(s) of protein fraction *Y* is not linked to the capacity to form the cobamide-containing enzyme from the apoenzyme. The apoenzyme is present in methionine-grown *E. coli* 3/62, as it is in extracts of strains W, PA15 and 121/176 which also have fraction *Y* activity when derived from organisms grown without added cobalamin.

It was found by visual observation that protein fraction *Y* caused the reduction of FAD to $FADH_2$ in the presence of $NADH_2$; $FADH_2$ did not, however, alone support methionine formation. Both ATP and reduced flavin are known to be required for the biosynthesis from cobalamin of dimethylbenzimidazolylcobamide coenzyme (Brady & Barker, 1961; Peterkofsky, Redfield & Weissbach, 1961); the coenzyme was therefore tested for ability to replace protein fraction *Y* plus ATP and the reducing system in the methylation of homocysteine, but it had no significant activity.

DISCUSSION

The results as a whole further strengthen the view that there are two enzymic mechanisms for the methylation of homocysteine in *E. coli*; they are set out in Scheme 1, which also takes into account the results of the two preceding papers (Foster *et al.* 1964*b*; Guest *et al.* 1964*a*).

The transfer of methyl groups specifically from N^5 -methyl- H_4 PtG₃ to homocysteine catalysed by preparations of enzyme *B* (cobalamin-independent pathway) showed no requirement for any addi-

Scheme 1. The two pathways for the methylation of homocysteine with serine as the ultimate donor of the C_1 unit. I, Cobalamin-dependent pathway; H_4 PtG₃ refers to either H_4 PtG or H_4 PtG₃. II, Cobalamin-independent pathway.

tional enzyme or cofactor other than Mg^{2+} ions. Although the degree of purification achieved for this enzyme was low, it is probable that only a single enzyme takes part in the reaction since the ability to catalyse methyl transfer parallels, during fractionation, the ability to complete the enzymic constitution of the cobalamin auxotroph (strain 121/176), which is almost certainly an organism with a single metabolic lesion; enzyme *B* is then a N^5 -methyltetrahydropteroyltriglutamate-homocysteine methyltransferase. It is, however, conceivable that after purification there remains present in excess in the active fraction a bound cofactor or associated enzyme other than one of those implicated in the cobalamin-dependent pathway.

The apparent simplicity of the cobalamin-independent pathway contrasts sharply with that dependent on cobalamin, where strict requirements were found for ATP, a reducing system containing FAD and a protein fraction additional to the previously recognized cobamide-containing enzyme. Two further major differences are the inability of enzyme *B* to use N^5 -methyl- H_4 PtG as substrate, and the absence of cobalamin from this enzyme (Foster *et al.* 1964*b*). The mechanisms of methyl transfer in the two systems appear therefore to differ radically; with enzyme *B* it is possible that the terminal glutamate residues of the triglutamate participate in labilizing the methyl group, a role taken over in the other pathway by a complex cobalamin system.

The cobalamin-dependent pathway studied here is apparently the same as that described briefly by Buchanan, Larrabee, Cathou & Rosenthal (1961) and Rosenthal & Buchanan (1962), who investigated *E. coli* 113/3, an auxotroph similar to strain 121/176 in responding to cobalamin or methionine. The enzyme fraction *D* of these authors is similar to protein fraction *Y* in its catalysis of the reduction of FAD. Exploratory experiments on the further purification of fraction *Y* suggested that this fraction has a necessary activity other than in $FADH_2$ formation. This has since been found to be the case, as reported briefly by Foster, Dilworth & Woods (1964*a*), who found that fraction *Y* also converts ATP plus methionine into *S*-adenosylmethionine; the latter is required in catalytic amounts for methyl transfer from N^5 -methyl- H_4 PtG to homocysteine. Evidence was also obtained that methyl groups from the folate are transferred to homocysteine via protein-bound *S*-adenosylmethionine and 'methylcobalamin' (the methyl analogue of the cobamide coenzymes); the latter is known to act as a methyl donor to homocysteine, a reaction catalysed by purified cobamide-containing enzyme and with no detectable cofactor requirements (Guest, Friedman, Woods & Smith, 1962; Guest,

Friedman, Dilworth & Woods, 1964*b*). A possible mechanism of the cobalamin-dependent transmethylation is fully discussed by Foster *et al.* (1964*a*) and Guest *et al.* (1964*b*); it takes into account also the present findings that free *S*-adenosylhomocysteine is a poor substrate and that there is no net formation of methionine from *S*-adenosylmethionine.

While the present work was in progress, several short reports have described the methylation of homocysteine by N^5 -methyl- H_4 PtG with enzymes from mammalian liver. The cofactors required (ATP, a reducing agent and Mg^{2+} ions) are similar to those noted above for the cobalamin-dependent pathway (Sakami & Ukstins, 1961; Mangum & Scrimgeour, 1962; Rooze, Sakami & Blair, 1962; Rohrbaugh, 1962).

SUMMARY

1. The two reactions by which the methyl group of N^5 -methyltetrahydrofolates is transferred to homocysteine by *Escherichia coli* have been further studied with purified preparations of the relevant enzymes.

2. Enzyme *B*, now characterized specifically as a N^5 -methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, requires only Mg^{2+} ions for full activity; Mn^{2+} ions are also active, but do not support optimum methionine formation. Enzyme *B*, at several stages of purification, completed the enzymic constitution of a methionine mutant with a single metabolic lesion, thus supporting the view that it is a single protein.

3. The cobamide-containing enzyme, which can use as substrate both N^5 -methyltetrahydropteroylmonoglutamate and -triglutamate, requires for activity the presence of another protein fraction (which may be complex) as well as ATP, Mg^{2+} ions, FAD and a $NADH_2$ -generating system. *S*-Adenosylhomocysteine replaces homocysteine as methyl acceptor to only a limited extent, and *S*-adenosylmethionine does not yield methionine even with unpurified extracts of *E. coli*. $FADH_2$, whose formation from FAD is catalysed by the accessory protein fraction, does not replace that fraction, the function of which is not yet clear.

4. The results further emphasize the radical difference in mechanism of the two transmethylation reactions.

We are grateful to the Nuffield Foundation for a grant towards the expenses of this work, which was also aided by grants to the Department from the Rockefeller Foundation and the U.S. Department of Health, Education and Welfare. J.R.G. and M.A.F. were Guinness Research Fellows in Microbiological Biochemistry; S.F. is indebted to the National Foundation (U.S.) for a Fellowship, and G.T. to the Juan March Foundation (Spain) for a Research Fellowship.

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Biochem. J. (1964), **92**, 504

Effect of Cysteine on Respiration and Catalase Synthesis by *Saccharomyces cerevisiae*

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(Received 16 September 1963)

Catalase synthesis can be induced in anaerobically grown cells of *Saccharomyces cerevisiae* by aeration (Chantrenne, 1954). In a study of the effect of various nitrogenous substances on the ability of anaerobically grown yeast cells to synthesize catalase, it was found that cysteine inhibited the catalase induction severely (Bhuvaneshwaran, Rege & Sreenivasan, 1961). In the present paper, the effect of cysteine on catalase synthesis and on respiration is examined in more detail.

MATERIALS AND METHODS

Yeast cultures and preparation of suspensions. The organism used was a brewing strain of *Saccharomyces cerevisiae* which was maintained by bi-weekly transfer on agar slants consisting of 2% (w/v) glucose, 1% (w/v) Bactopeptone and 0.3% yeast extract with 2% (w/v) agar.

For experimental purposes the organism was grown in a broth of the same composition but with the omission of agar for 20 hr. at 30° in stationary flasks. Practically anaerobic conditions were maintained by using 500 ml. conical flasks filled to the neck. Most of the yeast cells were at the bottom of the flask at the end of the incubation

period. The cells were harvested and washed twice with ice-cold distilled water by suspension and centrifuging at 10° and used at once.

The organism also grew well on a synthetic medium consisting (w/v) of NH₄Cl (1%), glucose (1%), KH₂PO₄ (1%), MgSO₄·7H₂O (0.01%) and CaCl₂ (0.01%). This medium after the omission of NH₄Cl served as the non-growth medium in aeration experiments; the pH was adjusted to 6.8.

Yeast cells (about 60–80 mg. dry wt.) were suspended in 10 ml. of this medium and were aerated in 50 ml. conical flasks by shaking in a reciprocating shaker at 60 oscillations/min. for 4 hr. A colorimeter calibrated in terms of dry weight facilitated the adjustment of yeast concentration. The cells were then separated by centrifugation, washed with distilled water and resuspended in 10 ml. of water. Catalase activity of the cells was determined after rupture by repeated freezing and thawing.

Assay of catalase. Catalase was assayed according to a procedure based on the titanium colour reaction for hydrogen peroxide (Patti & Bonnet Maury, 1953) and developed in this Laboratory by S. P. Manjrekar & A. Sreenivasan (unpublished work). The titanium reagent was prepared as follows: 1 g. of TiO₂ and 10 g. of K₂SO₄ were mixed and digested with 150 ml. of conc. H₂SO₄ for 5–6 hr. The digest was then cooled and diluted to 1.5 l. with distilled water. This served as the stock solution and could be stored at room temperature. The working solution was obtained by diluting the stock solution fivefold with 10% (v/v) H₂SO₄.

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