Two Enzymic Mechanisms for the Methylation of Homocysteine by Extracts of *Escherichia coli*

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Earlier work in this Laboratory has established conditions for the formation of methionine, from homocysteine plus serine or formaldehyde, by soluble enzyme systems extracted from *Escherichia coli*. A cofactor of the folic acid group* is essential, and with *E. coli* PA 15 an extract of the heated organisms, H_4PtG_3 and its N^5- and N^{10}-formyl derivatives are all effective. H_4PtG inhibits competitively the action of both H_4PtG_3 and the extract of heated *E. coli* (Szulmajster & Woods, 1960; Jones, Guest & Woods, 1961); such inhibition does not, however, occur if cobalamin is added either to the ultrasonic extracts or to the medium on which the organisms are grown (Kisliuk & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960). With ultrasonic extracts of *E. coli* 121/176 (an auxotroph requiring cobalamin or methionine for growth) cobalamin is essential even when H_4PtG_3 (or extract of heated *E. coli*) is present. Cobalamin acts after conversion into a complex factor (Kisliuk & Woods, 1960; Guest & Woods, 1960) which has been purified from strain PA 15 and shown to be a cobamide-containing enzyme (Foster, Jones & Woods, 1961*a*; Kisliuk, 1961). Analogues of cobalamin inhibit the formation of the cobamide-containing enzyme from cobalamin or 5,6-dimethylbenzimidazolylcobamide coenzyme, but are without effect on the activity of extracts of organisms grown in the presence of added cobalamin (Guest, 1960) and presumably possessing the cobamide-containing enzyme.

The enzymic synthesis of methionine from homocysteine and serine with H_4PtG as cofactor has also been studied in *E. coli* 113/3 (another cobalamin or methionine auxotroph) and found to require a cobamide-containing enzyme which is formed from cobalamin and an apoenzyme present in extracts of autotrophic organisms grown in the absence of cobalamin (Hatch, Takeyama & Buchanan, 1959; Hatch, Cathou & Larrabee, 1960; Takeyama & Buchanan, 1960).

Extracts of mammalian liver form methionine with H_4PtG (or its N^5-formyl derivative) as cofactor (Nakao & Greenberg, 1958; Stevens & Sakami, 1959); evidence was obtained by Wilmanns, Rücker & Jaenicke (1960) that H_4PtG itself acts as the hydrogen donor for the formation of the methyl group of methionine and N^5-methyldihydropteroylmonoglutamate (N^5-methyl-H_2PtG) was proposed as an intermediate. There was no requirement for cobalamin in these mammalian systems.

The present study was undertaken to clarify further the relationship between cobalamin and folic acid in the synthesis of methionine by *E. coli*. By using several strains of *E. coli* that grew with methionine but not with homocysteine, i.e. each unable to bring about the overall reaction, a successful search was made for further enzymes (apart from the cobamide-containing enzyme) that might take part. Evidence is presented for two distinct and alternative enzymic mechanisms for the methylation of homocysteine. One is catalysed by an enzyme (*A*) plus the cobamide-containing enzyme and the other by the enzyme *A* plus a third enzyme (*B*). H_4PtG is active in the first system, but not in the second, which has a specific requirement for H_4PtG_3. Brief reports of part of the present work have been made by Foster, Tejerina & Woods (1961*b*) and Guest & Woods (1962*a*).

While this work was in progress, an enzyme concerned in the methylation of homocysteine by extracts of *E. coli* 113/3 and absent from *E. coli* 205/2 (an auxotroph requiring *p*-aminobenzoic acid and methionine) was described by Hatch, Larrabee, Cathou & Buchanan (1961). Preparations of this enzyme catalysed the formation, from N^5N^{10}-methylene-H_4PtG and $NADH_2$, of an intermediate which was used for the methylation of homocysteine by preparations of the cobamide-containing enzyme in the presence of ATP, FAD, $NADH_2$ and Mg^{2+} ions (Larrabee & Buchanan, 1961).

MATERIALS AND METHODS

Organisms and enzyme-containing extracts. E. coli strain Pa 15 is an auxotroph requiring either serine or glycine for growth; it was originally obtained from Dr Barbara Wright and the parent strain was isolated from sewage (B. E. Wright, personal communication). *E. coli* strains 121/176 and 113/3 were both auxotrophs requiring methionine or

* 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^5N^{10}-methylene-H_4PtG.

cobalamin for growth, and were derived from the prototrophic strain W (Davis & Mingioli, 1950). *E. coli* strain 3/62 required methionine and did 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). All strains were maintained on slopes of nutrient agar (Oxo Ltd., London, E.C. 4), subcultured monthly, incubated 16 hr. at 37° and stored at 4°.

Organisms were grown on solid defined media in enamelled trays as described by Guest *et al.* (1960) when required for the preparation of ultrasonic extracts, or according to Szulmajster & Woods (1960) when used for the preparation of acetone-dried powders. The medium was supplemented with glycine (10 mM) for the growth of strain PA 15, and with DL-methionine (0·5 mM) for the growth of all other strains. Cobalamin (15 μm-moles/500 ml. of medium), when present, was added to the surface of each tray with the inoculum.

Ultrasonic 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) both for the preparation of suspensions of organisms before ultrasonic treatment and for the dialysis of the extracts. Extracts of acetone-dried organisms (strain PA 15) were prepared as described by Jones *et al.* (1961). Both types of extract were sometimes treated with Dowex 1 resin (X8; Cl^- form; 100–200 mesh) by the method of Kisliuk & Woods (1960). The protein and nucleic acid content of extracts was determined spectrophotometrically according to Layne (1957).

An extract of heated *E. coli*, containing natural forms of folic acid, was prepared from acetone-dried organisms of strain PA 15 (grown in the absence of added cobalamin) as described by Guest *et al.* (1960).

All extracts were stored at −20° and lost no activity over a period of 6 months.

Test for methionine formation. The conditions used for testing methionine synthesis were based on previous work in this Laboratory. The basal reaction mixture 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; $MgSO_4$, 5 mM; NAD, 0·5 mM; FMN, 0·25 mM; pyridoxal phosphate, 0·5 mM; 2-mercaptoethanol, 4 mM; enzyme preparation equivalent to 5–6 mg. of protein/ml. In some experiments glucose was replaced by a $NADH_2$-generating system consisting of ethanol (10 mM) and crystallized yeast alcohol dehydrogenase (30 μg./ml.). In later work, FMN was replaced by FAD (0·25 mM) and the pyridoxal phosphate concentration decreased to 0·1 mM. The source of folic acid cofactor was the extract of heated *E. coli* (equivalent to 14 mg. dry wt. of original organisms/ml. of reaction mixture), H_4PtG_3 (0·05 mM) or H_4PtG (0·5 mM).

The complete reaction mixtures were incubated at 37° in an atmosphere of H_2 and the enzymic reaction was terminated by heating for 3 min. at 100°. Incubation was usually for 1·5 hr. (3 hr. when H_4PtG was used). 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.

Cobamide-containing enzyme. This was assayed as described by Foster *et al.* (1961*a*) through its ability to promote methionine synthesis (by extracts of acetone-dried organisms of strain PA 15 grown without cobalamin) in a reaction mixture containing H_4PtG as folic acid coenzyme.

Preparations of purified cobamide-containing enzyme were made from acetone-dried organisms of strain PA 15 grown in the presence of cobalamin by a modification of the method of Foster *et al.* (1961*a*). Crude extracts were treated with $CaCl_2$, subjected to isoelectric precipitation and treated with protamine sulphate as described originally, but in the column chromatography of the enzyme with protamine on Celite 545 the removal of inert protein with 120 mM-potassium phosphate buffer, pH 7·8, was continued until 500 ml. (instead of 120 ml.) had passed through before eluting the enzyme with 160 mM-potassium phosphate buffer, pH 7·8. The remaining four steps of the purification were omitted. This procedure gave a preparation with the same degree of purification (150-fold), but in a yield (6% overall) double that of the original method. Batches of purified enzyme were used on the day the preparation was completed.

Assay of cobalamin. Materials were first suspended or dissolved in 20 mM-potassium phosphate, pH 6·5, containing potassium cyanide (1–10 mM) and autoclaved (for 10 min. at 115°). After digestion for 48 hr. at 25° under aseptic conditions with papain (final concn. 300 μg./ml.; sterilized by Seitz-filtration), the mixture was adjusted to pH 4·5 with acetic acid and heated at 100° for 10 min. The supernatant fluid, after centrifuging, was used for the assay of cobalamin with *Euglena gracilis* var. *bacillaris* by the procedure of Kisliuk & Woods (1960). Growth was assessed in an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with red filter no. 608, or by measuring (in a Unicam SP. 600 spectrophotometer) the extinctions at 420 mμ and 670 mμ of the pigments extracted with ethyl acetate (Epstein & Weiss, 1960). Standard cobalamin was prepared according to Foster *et al.* (1961*a*); none of the test materials influenced the response of the organism to added cobalamin. Results are corrected for the recovery (90–100%) of cobalamin added to control tubes in the preparation of the test samples. With purified cobamide-containing enzyme the elaborate treatment of samples to release cobalamin was unnecessary and the more rapid (though less sensitive) procedure of Foster *et al.* (1961*a*) with *E. coli* 113/3 as assay organism was used.

Chemicals. H_4PtG was prepared as described by Kisliuk & Woods (1960) and stored as the solid under H_2. H_4PtG_3 was prepared according to the method of Jones *et al.* (1961) from pteroyl-γ-glutamyl-γ-glutamylglutamic acid (PtG_3) (given by Dr E. L. R. Stokstad). DEAE-cellulose (DE 50) and CM-cellulose (CM 70) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.; triethylaminoethylcellulose (TEAE-cellulose) (capacity 0·6 m-equiv./g.) was a product of Serva Entwicklungslabor, Heidelberg, Germany. Alumina C_γ gel was prepared according to the method of Bauer (1945) and contained 60 mg. dry wt. of solids/ml. Protamine sulphate (from herring roe) was obtained from L. Light and Co. Ltd., crystallized yeast alcohol dehydrogenase from C. F. Boehringer und Soehne, Mannheim, Germany, crystallized trypsin from Armour Laboratories, Hampden Park, Eastbourne, Sussex, and twice-recrystallized papain from British Drug Houses Ltd., Poole, Dorset, who also supplied crystallized cobalamin; the latter was about 90% pure by spectrophotometric assay. The anilide analogue of cobalamin was a gift from Dr E. Lester Smith. 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, U.S.A.) were also commercial products.

RESULTS

Activation by the cobamide-containing enzyme of extracts of a cobalamin/methionine auxotroph

Ultrasonic extracts of strain 121/176 methylate homocysteine with H_4PtG as cofactor only if cobalamin is present; the latter is presumed to act after conversion into a cobamide-containing enzyme (Guest & Woods, 1960). Purified cobamide-containing enzyme (from strain PA 15 grown in the presence of cobalamin) has now been shown to activate the ultrasonic extract of strain 121/176 in the presence of H_4PtG, H_4PtG_3 or the extract of heated *E. coli* (Table 1). In the one case tested (H_4PtG), activation by the cobamide-containing enzyme was more than ten times as efficient as that by cobalamin when activities were compared in terms of the cobalamin content of the enzyme. Further, the action of the cobamide-containing enzyme was not inhibited by the anilide analogue of cobalamin at a concentration that completely inhibited the action of cobalamin itself (Table 1).

Methionine synthesis by mixtures of extracts from different strains

Ultrasonic extracts of strain 3/62 (an auxotroph requiring methionine for growth), and of strains 121/176 and 113/3 (responding either to cobalamin or methionine), harvested after growth with methionine, each failed to synthesize methionine when the folic acid cofactor was either the extract of heated *E. coli* or H_4PtG_3 (Table 2). However, mixtures of such extracts derived from strains 3/62 and 121/176 (or from strains 3/62 and 113/3) were highly active with either the extract of heated *E. coli* or H_4PtG_3. With H_4PtG no single extract or combination of two extracts was active. The ability to use the monoglutamate as cofactor was, with strains 121/176 and 113/3 (and mixtures containing them), dependent on the addition of cobalamin (Table 2, last column); even then the synthesis of methionine was relatively low. Cobalamin did not activate extracts of strain 3/62 under any conditions tested.

It is clear that the metabolic lesion in strain 3/62 is distinct from that in strains 121/176 and 113/3, and that the defect in the latter strains is probably the same.

Two further important points emerge from the results obtained with mixed extracts of strains 3/62 and 121/176. First, methionine is not formed when the folic acid cofactor is H_4PtG and hence strain 3/62 cannot be providing the cobamide-containing enzyme. Secondly, the synthesis obtained with H_4PtG_3 or the extract of heated *E. coli* as cofactor is about ten times as great as that obtained with 121/176 extract and a cofactor system of H_4PtG plus cobalamin.

Maximum methionine synthesis by mixtures of extracts of strains 3/62 and 121/176 (or 113/3) was obtained with equal amounts of protein from each strain (Fig. 1). Since the extract of strain 113/3 behaved in a quantitatively similar manner to that from strain 121/176, it appears that not only are the strains identical with respect to the enzyme supplied

Table 1. *Activation of extract of* Escherichia coli *strain* 121/176 *by cobalamin or cobamide-containing enzyme*

The extract of strain 121/176 was incubated for 3 hr. in complete reaction mixture (final vol. 1 ml.) with the folate shown. Purified cobamide-containing enzyme (6 μg. of protein), when present, was equivalent to a cobalamin concn. of 1·8 μmM; further additions were as shown. —, Not tested.

Source of folic acid	Additions	L-Methionine formed (μm-moles): Cobamide-containing enzyme absent	L-Methionine formed (μm-moles): Cobamide-containing enzyme present
Extract of heated *E. coli*	None	20	430
H_4PtG_3	None	20	425
H_4PtG	None	10	375
H_4PtG	Cobalamin (1·8 μmM)	35	—
H_4PtG	Cobalamin (37 μmM)	250	—
H_4PtG	Vitamin B_{12} anilide analogue (6 μM)	10	375
H_4PtG	Cobalamin (37 μmM) + vitamin B_{12} anilide analogue (6 μM)	10	—

Table 2. *Methionine synthesis by mixed extracts of mutant strains of* Escherichia coli

The complete reaction mixture containing the folate shown was incubated for 1·5 or 3 hr. with enzymic extract equivalent to a total of 5 mg. of protein/ml.; when mixtures of extracts were used half was derived from each strain. Cobalamin (0·37 μM) was added as indicated.

Source of folic acid	L-Methionine formed (μm-moles/mg. of protein/hr.): Extract of heated *E. coli*	H_4PtG_3	H_4PtG	H_4PtG + cobalamin
Strain used as source of extract				
3/62	2	2	0	1
121/176	2	2	1	10
113/3	2	2	1	10
3/62 + 121/176	95	103	1	8
3/62 + 113/3	92	100	3	8
121/176 + 113/3	3	—	1	—

by them in the present test system (i.e. the enzyme missing from strain 3/62), but also that both lack the enzyme which can be supplied by strain 3/62. Strain 121/176 was chosen for further work since more background information about it in relation to methionine synthesis was available in this Laboratory. The results of Fig. 1 were quantitatively reproduced with extracts stored for 6 months at $-15°$.

Further details of the substrate and cofactor requirements of the reaction as catalysed by an equipartite mixture (in terms of protein) of extracts of strains 3/62 and 121/176 were next investigated. Either H_4PtG_3 or the extract of heated *E. coli* was essential; half-maximal synthesis was obtained with 4·3 μM-H_4PtG_3 or with the extract of heated *E. coli* at a final concentration equivalent to 1·1 mg. dry wt. of organisms/ml. The cofactor activity of either the extract of heated *E. coli* or H_4PtG_3 was decreased to about one-third in the presence of H_4PtG at 20 times the concentration of H_4PtG_3 (compare lines 1 and 3 in Table 3). Other requirements, with either the extract of heated *E. coli* or H_4PtG_3 as cofactor, were similar to those reported for strain PA 15 by Guest *et al.* (1960). Negligible synthesis occurred when homocysteine, serine or pyridoxal phosphate was omitted, and the addition of cobalamin was without effect (Table 3). Maximum synthesis required Mg^{2+} ions, NAD, glucose, ATP and anaerobic conditions.

Glucose was not needed if a $NADH_2$-generating system was added (Table 3). In previous work in this Laboratory with strain PA 15 a requirement for FMN had been observed (Foster *et al.* 1961*a*; Guest & Woods, 1962*b*). Under the present conditions FAD increased synthesis by about 30 % (Table 3). When the mixed extract (after dialysis and treatment with Dowex 1 resin) was filtered through Sephadex G-50 (Kisliuk, 1960) the effect was sharpened and the omission of FAD resulted in a 70 % decrease in methionine synthesis; FMN only partially replaced FAD.

Since extracts of either strain 3/62 or 121/176 contain the enzyme (serine hydroxymethyltransferase) catalysing the transfer of the hydroxymethyl group of serine to H_4PtG or H_4PtG_3, the results obtained so far established an *a priori* case that the overall transfer (with concomitant reduction) of the hydroxymethyl group from reduced folic acid to homocysteine, in the absence of added cobalamin, requires at least two enzymes. One of

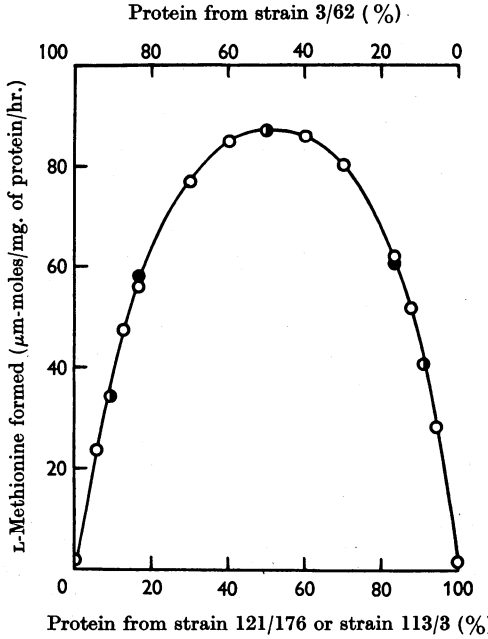


Fig. 1. Synthesis of methionine by mixtures (in various proportions) of cell-free extracts derived from different auxotrophs of *E. coli*. Dialysed extracts containing protein in the relative amounts shown from strains 3/62 and 121/176 (○) or strains 3/62 and 113/3 (●) were incubated in the basal reaction mixture, with the extract of heated *E. coli* as cofactor.

Table 3. *Requirements for methionine synthesis by mixed extracts of* Escherichia coli *strains* 3/62 *and* 121/176

A mixture of dialysed and Dowex 1-treated extracts from strain 3/62 and strain 121/176 (each 2·5 mg. of protein/ml.) was incubated with the extract of heated *E. coli* or H_4PtG_3 in the usual reaction mixture with the omissions or additions shown. The $NADH_2$-generating system contained ethanol (10 mM) and crystallized alcohol dehydrogenase (30μg./ml.).

Modifications to reaction mixture: Omission	Addition	L-Methionine formed (μm-moles/mg. of protein/hr.): Source of folic acid: Extract of heated *E. coli*	H_4PtG_3
None	None	92	93
None	Cobalamin (0·37 μM)	93	93
None	H_4PtG (1 mM)	27	32
Homocysteine	None	0	5
Serine	None	2	0
$MgSO_4$	None	71	70
NAD	None	71	70
Pyridoxal phosphate	None	8	19
FAD	None	68	68
ATP	None	64	36
Glucose	None	46	10
Glucose	$NADH_2$-generating system	—	88
H_2	Air	72	71

these (provisionally called enzyme *A*) is missing from strain 3/62 but present in strain 121/176. The other (enzyme *B*) is present in strain 3/62 but absent from 121/176; since extracts of the latter strain can bring about the total reaction if supplemented with the cobamide-containing enzyme it is probable that enzyme *B* has the same function as the cobamide-containing enzyme. However, if it were the cobamide-containing enzyme it would be expected that H_4PtG would be an effective cofactor (as with 121/176 extract plus cobamide-containing enzyme; Table 1) whereas only the triglutamate form (H_4PtG_3) or the extract of heated *E. coli* was active (Table 2).

Preparation of enzyme A from Escherichia coli *strain* 121/176

Detection and assay. The addition of a relatively small proportion (5–20 %) of 121/176 extract enabled 3/62 extract to form measurable amounts of methionine with the extract of heated *E. coli* as folic acid cofactor (Fig. 1). The response obtained by adding graded small amounts (0–2 mg. of protein) of 121/176 extract (containing enzyme *A*) to a standard amount (10 mg. of protein) of 3/62 extract was almost linear (Fig. 2), and made possible a valid assay for enzyme *A*. The shape of the curve was little influenced by increasing the time of incubation up to 4 hr. One unit of enzyme *A* activity is defined as that amount which causes 10 mg. of extracted protein of strain 3/62 to synthesize 500 μm-moles of L-methionine in 1·5 hr. with the extract of heated *E. coli* as cofactor. Crude extracts of strain 121/176 had a specific activity of 0·8–1·2 units/mg. of protein.

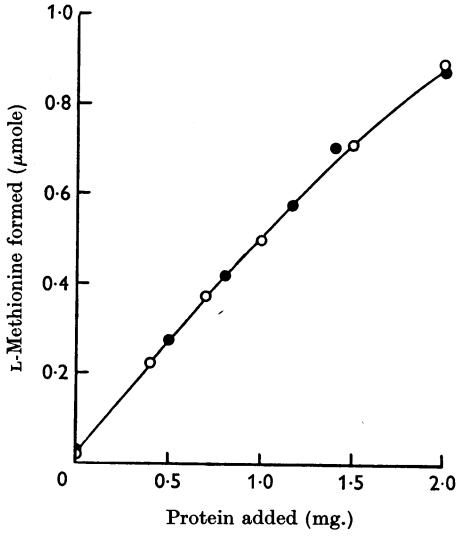


Fig. 2. Assay of enzymes *A* and *B*. Extracted protein of *E. coli* strain 121/176 (●) or strain 3/62 (○) was added as shown to 10 mg. of extract protein of strain 3/62 (—●—) or strain 121/176 (—○—). The extract of heated *E. coli* was used as cofactor in the basal reaction mixture (final vol. 2 ml.); incubation was for 1·5 hr.

Crude extracts of other strains of *E. coli* (PA 15, B, 518 and K 12) also contained enzyme *A*. Since enzyme *B* is also present in these strains, such extracts, in the amounts used in the assay for enzyme *A*, themselves catalysed the formation of some methionine; this value was measured separately in a control and subtracted. The specific activities were then in the range 0·15–0·30 unit/mg. of protein, i.e. significantly less than for strain 121/176. Consequently, the latter strain was chosen as a source of enzyme *A* for purification.

Purification. All operations were conducted at 0–4° and the buffer solutions used contained 2-mercaptoethanol (7 mM). Crude undialysed extract of strain 121/176 was diluted to 20 mg. of protein/ml. with 20 mM-potassium phosphate buffer, pH 7·8, and treated with protamine sulphate (20 mg./ml. in the same buffer), 0·8 ml./10 ml. of extract being used. After standing for 30 min. and centrifuging at 24 000*g* for 10 min., the supernatant fluid contained 50–60 % of the protein, 80–100 % of the enzyme activity and only 8 % of the nucleic acid of the initial extract. This material was fractionated by the progressive and slow addition of saturated ammonium sulphate solution which had been previously brought to pH 7·8 with ammonia. The precipitates obtained at 42 and 50 % saturation respectively had no enzyme activity and were discarded. The precipitate formed between 50 and 70 % saturation was collected by centrifuging, drained as free as possible of fluid, dissolved in the minimum volume of 20 mM-potassium phosphate, pH 7·0, and dialysed for 16 hr. against two changes of the same buffer. The final solution (fraction I) contained about 15 % of the protein and 55 % of the activity of the initial extract; it was stable when stored at −20°.

No increase in specific activity resulted from attempts at further purification of fraction I (*a*) by the use of calcium phosphate or alumina C_γ gels, (*b*) by zone electrophoresis, or (*c*) by chromatography on DEAE-cellulose or on CM-cellulose. Chromatography on TEAE-cellulose was, however, successful in small-scale experiments. Fraction I was diluted to 20 mg. of protein/ml. and dialysed against 5 mM-potassium phosphate, pH 6; samples (1 ml.) were passed through a column (1 cm.^{2} × 5 cm.) of the cellulose equilibrated with 5 mM-potassium phosphate, pH 6·0. The initial effluent (fraction II) contained 45 % of the enzyme and only 1·3 % of the protein of fraction I, and had a specific activity of 80 units/mg. of protein. The pro-

Table 4. *Purification of enzyme* A *from extract of* Escherichia coli *strain* 121/176

Details of the experimental procedure are given in the text.

	Volume (ml.)	Total protein (mg.)	Total enzyme activity (units)	Specific activity (units/mg. of protein)	Overall yield of enzyme (%)
Crude extract	285	6470	7900	1·2	100
Supernatant after protamine treatment	268	2850	6800	2·4	86
Ppt. with 50–70 % saturated $(NH_4)_2SO_4$ (fraction I)	15	880	4100	4·6	52
Eluent from Celite column	85	12·1	540	45	6·8
Ppt. formed on dialysis	—	3·6*	345*	96	4·4
Final phosphate (80 mM) extract (fraction III)	10	1·2	93	78	1·2

* Calculated by difference.

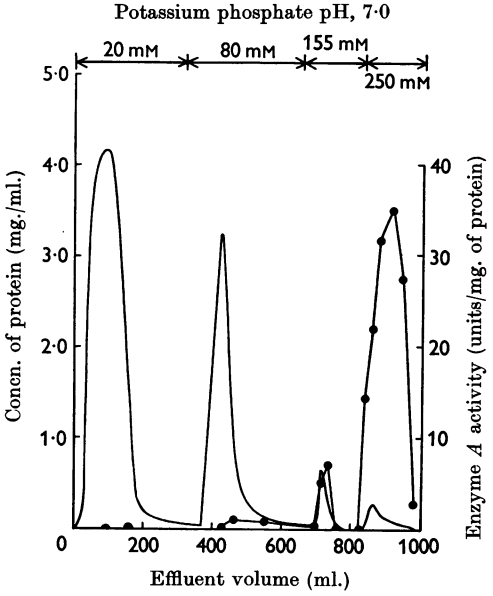


Fig. 3. Chromatography of enzyme *A* on Celite 545. Dialysed fraction I (see the text) (90 ml. containing 900 mg. of protein and 3900 units of enzyme *A*) was chromatographed as described in the text; 10 ml. fractions were collected. Protein (—) was estimated for each fraction; points are omitted for clarity. ●, Specific activity of enzyme *A*.

cedure was reproducible and was used on a few occasions to provide enzyme of high specific activity for metabolic experiments; it was not, however, effective on a larger scale.

Successful fractionation of larger amounts of fraction I was achieved by applying the principles of the method devised by Foster *et al.* (1961*a*) for the purification of the cobamide-containing enzyme, i.e. precipitation of the enzyme with protamine, re-elution of the enzyme–protamine complex from the precipitate and chromatography on Celite 545. Dialysed fraction I (900 mg. of protein in 90 ml. of 20 mM-potassium phosphate buffer, pH 7·0) was treated with 4·5 ml. of protamine sulphate (20 mg./ml.) and with 2 g. of Celite 545. The suspension was poured into a column (4 cm.^{2} × 2·5 cm.) and the effluent run through a larger Celite 545 column (6·5 cm.^{2} × 30 cm.) previously washed with the 20 mM buffer. Passage of 80 mM-potassium phosphate buffer, pH 7·0, through both columns transferred enzyme *A* to the larger column; after the upper column had been discarded and the removal of some protein from the lower one with 155 mM-potassium phosphate buffer, pH 7·0, the enzyme was eluted with 250 mM-potassium phosphate buffer, pH 7·0. A typical chromatographic separation is shown in Fig. 3. The pooled active fractions from the Celite column contained 25–45 units of enzyme *A* activity/mg. of protein, and when dialysed against 10 mM-potassium phosphate buffer, pH 7·0, yielded a precipitate containing the enzyme. Successive extraction of this with 10 ml. portions of 20 mM-, 50 mM- and 80 mM-potassium phosphate buffer, pH 7·0, resulted in a final solution containing 80 units of enzyme/mg. of protein. After the addition of serum albumin (5 mg./ml.) the product (fraction III) was stored at −20° for up to 3 weeks before use. Data at different stages of purification by the larger-scale procedure are given in Table 4.

The active material in crude extracts of strain 121/176 was presumed initially to be an enzyme because it was non-diffusible and thermolabile; 50 % of the activity was lost on heating for 5 min. at 45°, or 100 % at 55°. Fraction II was stable only within the pH range 6–8; 50 % of the activity was lost in 1 hr. at pH 5 or pH 9. The most purified material (fraction III) had a 280 mμ:260 mμ extinction ratio of 1·6 and its activity was destroyed on digestion with crystalline trypsin. The catalytic nature of the purified protein is clear since, during assay, it promoted the synthesis of 4–6 times its own weight of L-methionine.

Preparation of enzyme B *from* Escherichia coli *strain* 3/62

Detection and assay. The enzyme was assayed by a method analogous to that used for enzyme *A*, i.e. by measurement of the amount of methionine syn-

thesized when small graded amounts of the preparation were added to a standard quantity (excess) of enzyme *A* provided by extract of strain 121/176; the response was again almost linear in the range used (Fig. 2). One unit of enzyme *B* was taken as the amount that caused 10 mg. of extracted protein of strain 121/176 to synthesize 500 μm-moles of L-methionine in 1·5 hr., with the extract of heated *E. coli* as cofactor.

Purification. Crude extracts of strain 3/62 contained 0·8–1·2 units of enzyme *B*/mg. of protein; extracts of other strains of *E. coli* (PA 15, B, 518 and K 12) contained less enzyme *B*, and strain 3/62 was therefore chosen for preparative work. Crude undialysed extract (50 ml.; 20 mg. of protein/ml.) was treated with protamine sulphate (8·75 ml.; 20 mg./ml. in 20 mM-potassium phosphate buffer, pH 7·8); over 90 % of the nucleic acid and 60 % of the protein was removed without loss of enzyme activity (Fig. 4). The supernatant fluid after centrifuging was submitted to column chromatography on DEAE-cellulose equilibrated with 20 mM-potassium phosphate buffer, pH 7·8; a salt gradient up to 590 mM-potassium chloride in 120 mM-potassium phosphate buffer, pH 7·8, was used for elution (Fig. 5). Reproducible results were not obtained with batches of the cellulose that had been regenerated after use in other experiments. The pooled fractions of highest specific activity were dialysed overnight against two changes of 5 mM-potassium phosphate buffer, pH 7·8, and then diluted with the same buffer so as to decrease the protein content to 0·7 mg./ml. Some inactive protein was removed by adsorption on to alumina C_γ gel (0·75 ml. of gel/100 ml. of solution) before adsorption of the active protein on to the same gel (5 ml. of gel/100 ml. of the supernatant from the first gel treatment). The enzyme was eluted with two portions (50 ml.) of 20 mM-potassium phosphate, pH 7·8; the product at this stage had a specific activity of 10–15 units of enzyme *B*/mg. of protein. The gel eluate could be concentrated without loss of activity by adsorbing the protein on to a column (1·5 cm.^{2} × 1·5 cm.) of DEAE-cellulose (equilibrated with 20 mM-potassium phosphate, pH 7·8) and eluting it with 10 ml. of 590 mM-potassium chloride in 120 mM-potassium phosphate buffer, pH 7·8. The product was stored at 0–4°. The results at various stages of purification are given in Table 5.

Further fractionation of the product by precipitation with ammonium sulphate, by isoelectric precipitation, by chromatography on CM-cellulose or by adsorption and elution from calcium phosphate gel did not increase the relatively low degree of purification achieved.

Properties. The material activating 121/176 extract was assumed to be enzymic since it was non-diffusible and thermolabile; 50 % of the

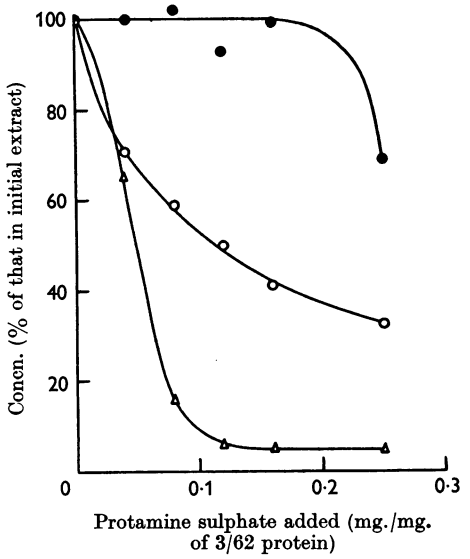


Fig. 4. Protamine treatment of extract of *E. coli* 3/62. Protamine sulphate was added as shown to samples of the crude extract. After standing at 0° for 30 min. and centrifuging, the supernatant fluid was analysed. Values are given as percentages of those for the initial material. ○, Concn. of protein; ●, concn. of enzyme *B*; △, concn. of nucleic acid.

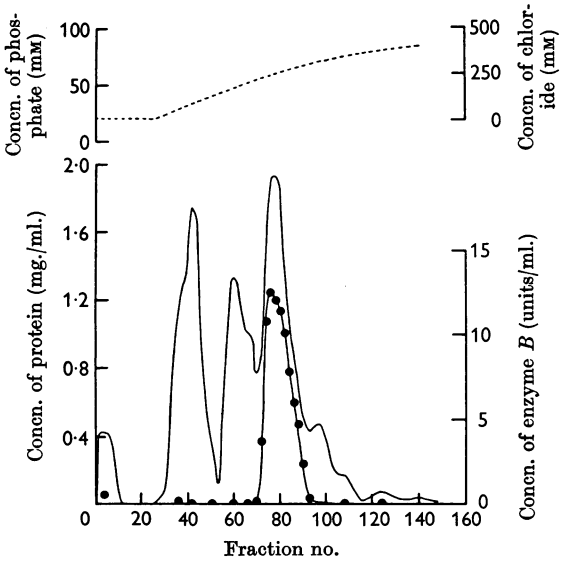


Fig. 5. Chromatography of enzyme *B* on DEAE-cellulose. The supernatant fluid after protamine treatment (52 ml. containing 380 mg. of protein and 1300 units of enzyme *B*) was chromatographed on DEAE-cellulose as described in the text; 5 ml. fractions were collected. Protein (—) was measured for each fraction; points are omitted for clarity. ●, Enzyme *B* concn.; - - - -, salt gradient.

Table 5. *Purification of enzyme* B *from extract of* Escherichia coli *strain* 3/62

Details of the experimental procedure are given in the text.

	Volume (ml.)	Total protein (mg.)	Total enzyme activity (units)	Specific activity (units/mg. of protein)	Overall yield of enzyme (%)
Crude extract	50	1150	1435	1·2	100
Protamine supernatant	55	403	1410	3·5	98
DEAE-cellulose fraction	75	120	1020	8·5	71
Dialysed DEAE-cellulose fraction	65	116	990	8·5	69
Alumina C_γ-gel supernatant (first treatment)	135	78	773	9·9	54
Alumina C_γ-gel eluent (after second treatment)	110	27	334	12·4	23
DEAE-cellulose concentrate	20	22	272	12·4	19

Table 6. *Sequence of action of enzymes* A *and* B

The extract of the indicated strain (5 mg. of protein/ml.) was first incubated anaerobically for 1·5 hr. with the substrate shown in a reaction mixture containing: glucose (20 mM), ATP (5 mM), $MgSO_4$ (5 mM), FMN (0·25 mM), NAD (0·5 mM), ethanol (10 mM), alcohol dehydrogenase (30 μg./ml.) and 2-mercaptoethanol (4 mM) in 120 mM-potassium phosphate, pH 7·8. After heating for 7 min. at 65°, the mixture was incubated for a further 1·5 hr. with extract (5 mg. of protein/ml.) of the strain and substrate shown. The concn. of the substrates used was homocysteine (6·7 mM) and, as sources of the N^5N^{10}-methylenetetrahydrofolates, formaldehyde (3·3 mM) plus either H_4PtG_3 or H_4PtG (3·3 mM).

Expt. no.	First incubation: Extract	First incubation: Substrate	Second incubation: Extract	Second incubation: Substrate	L-Methionine formed (μm-moles)
1	121/176	N^5N^{10}-Methylene-H_4PtG_3	3/62	Homocysteine	120 (180*)
	3/62	N^5N^{10}-Methylene-H_4PtG_3	121/176	Homocysteine	10
	121/176	Homocysteine	3/62	N^5N^{10}-Methylene-H_4PtG_3	15
	3/62	Homocysteine	121/176	N^5N^{10}-Methylene-H_4PtG_3	25
2	121/176	N^5N^{10}-Methylene-H_4PtG	3/62	Homocysteine	15
	121/176	N^5N^{10}-Methylene-H_4PtG	Cobamide-containing enzyme†	Homocysteine	460 (500*)

* Values obtained when the heating step was omitted.
† Provided as an extract of strain 3/62 grown with cobalamin.

activity of crude extracts was lost on heating for 5 min. at 48°, or 100 % at 55°. The purified material had a 280 mμ:260 mμ extinction ratio of 1·4, and was stable only in the pH range 7–8. The activity was lost on digestion with crystallized trypsin or papain. The enzyme, when purified, was unstable to repeated freezing and thawing, two cycles being sufficient to destroy half of the activity. The low degree of purification achieved so far has prevented the formal demonstration of a catalytic function of enzyme *B*.

Formation of intermediates

Evidence was first sought on the order in which enzymes *A* and *B* act. Two-step test systems were used in which a source of one enzyme was first incubated with either homocysteine or N^5N^{10}-methylene-H_4PtG_3 (in substrate concentration, provided as an equimolar mixture of formaldehyde and H_4PtG_3). Subsequently, after heating under conditions known to destroy the first enzyme, a source of the other enzyme and the other substrate were added and the incubation was continued.

Incubation of 121/176 extract (containing enzyme *A*) in a reaction mixture containing N^5N^{10}-methylene-H_4PtG_3, but no homocysteine or serine, yielded a product which gave rise to methionine on the subsequent addition of homocysteine and an extract (of strain 3/62) containing enzyme *B* (Expt. 1 in Table 6). No methionine was formed if either step was carried out at 0° or if the two enzyme preparations were incubated in any other order with either substrate (Expt. 1 in Table 6). The product formed by enzyme *A* from N^5N^{10}-methylene-H_4PtG_3 was subsequently stable to heating for 7 min. at 65° since only slightly more methionine was formed if the heat treatment before the second incubation was omitted.

The purified preparation of enzyme *A* described above effectively replaced the crude extract of strain 121/176 for the first stage of the reaction, and the intermediate formed gave rise to methionine on further incubation with homocysteine and either a purified preparation of enzyme *B* or the crude extract of strain 3/62 from which it was derived (Table 7). Although the degree of purification of

the two enzymes was not high, these results diminish the possibility that a further enzyme or enzymes is required for the overall reaction.

In similar experiments, N^5N^{10}-methylene-H_4PtG was substituted for the triglutamate derivative as direct source of the C_1 residue. There was no significant formation of methionine with mixtures of either crude (Table 6) or purified (Table 7) preparations of enzymes *A* and *B*, or in systems in which the source of one enzyme was crude and the other purified; the failure with the crude preparations is therefore unlikely to be due to the presence of an inhibitory factor. There was, however, considerable methionine formation when a source of the cobamide-containing enzyme was added during the second stage of the incubation (Expt. 2 in Table 6). To make conditions as comparable as possible the cobamide-containing enzyme was provided as an extract of strain 3/62 itself, but from organisms grown in the presence of cobalamin; the extract thus contained, besides cobamide-containing enzyme, all the materials present in the normal 3/62 extract used as source of enzyme *B*. It is concluded that enzyme *A* can also form from N^5N^{10}-methylene-H_4PtG a heat-stable intermediate, but that the further reaction of this with homocysteine is not catalysed by enzyme *B*, but only by the cobamide-containing-enzyme system.

Distinction between the cobamide-containing enzyme and enzyme B

It is clear from the work described above that enzyme *B* is sharply differentiated from the cobamide-containing enzyme in that only H_4PtG_3 is effective with the former whereas the monoglutamate is also active with the latter. However, since, when H_4PtG_3 (or the extract of heated *E. coli*) is used as cofactor, 121/176 extracts can form methionine if supplemented either with enzyme *B* or with the cobamide-containing enzyme, it was important to obtain further evidence of difference between the two enzymes.

The purified enzyme *B* preparation, even after treatment to release bound cobalamin, had barely detectable activity in promoting the growth of *Euglena gracilis* and was at the extreme lower limit of the assay for cobalamin. Assuming such activity to be due to cobalamin, enzyme *B* contained at most less than one-hundred-thousandth as much cobalamin as the cobamide-containing enzyme, i.e. 45 $\mu\mu$g./mg. of protein compared with the value of 0·55 μg. reported for the purified cobamide-containing enzyme by Foster *et al.* (1961*a*); both values relate, of course, to the present state of purification of the two enzymes. Further, purified enzyme *B* had no cobamide-containing-enzyme activity when assayed in the usual way with extracts of acetone-dried organisms of strain PA 15 grown in the absence of added cobalamin and with H_4PtG as cofactor.

The non-identity of the two enzymes was also made clear by the titration with protamine sulphate of an extract of strain 3/62 prepared from organisms grown in the presence of cobalamin and thus possessing both the cobamide-containing enzyme and enzyme *B*. The cobamide-containing enzyme can be assayed separately from enzyme *B* because it is active with H_4PtG as cofactor whereas enzyme *B* is not. The precipitation of the cobamide-containing enzyme, which required relatively small amounts of protamine sulphate, followed a sigmoid curve (Fig. 6). Any assay for enzyme *B* (which requires H_4PtG_3) will, however, also assay the cobamide-containing enzyme, since the latter is also active with H_4PtG_3. The precipitation of enzyme *B* from a source free from the cobamide-containing enzyme requires larger amounts of protamine sulphate (Fig. 4) than does the cobamide-containing enzyme.

Table 7. *Formation of methionine by purified preparations of enzymes* A *and* B

Purified enzyme *A* (fraction III; 24 μg./ml.) or 121/176 extract (5 mg. of protein/ml.) was first incubated anaerobically for 1·5 hr. with formaldehyde (3·3 mM), plus either H_4PtG_3 or H_4PtG (3·3 mM), serum albumin (5 mg./ml.) and the reaction mixture given in Table 6. After heating for 7 min. at 65°, the mixture was incubated a further 1·5 hr. with homocysteine (6·7 mM) and either purified enzyme *B* (75 μg./ml.) or extract of strain 3·62 (5 mg. of protein/ml.)

Substrate for first incubation	Source of enzyme: In first incubation	Source of enzyme: In second incubation	L-Methionine formed (μm-moles)
N^5N^{10}-Methylene-H_4PtG_3	121/176 extract	Enzyme *B*	145
	Enzyme *A*	3/62 extract	140 (175*)
	Enzyme *A*	Enzyme *B*	140
	None	3/62 extract	0
None	Enzyme *A*	3/62 extract	20
N^5N^{10}-Methylene-H_4PtG	121/176 extract	Enzyme *B*	20
	Enzyme *A*	3/62 extract	20 (25*)
	Enzyme *A*	Enzyme *B*	25

* Values obtained when the heating step was omitted.

When both enzymes are present in the same extract a diphasic precipitation curve would be expected, the first part corresponding to the curve for the cobamide-containing enzyme and the second to the later precipitation of enzyme *B*. Such a curve was, in fact, obtained (Fig. 6).

Purified cobamide-containing enzyme was stable for at least 2 hr. at 0° in 4·5 M-ammonium sulphate at pH 7·8, but protamine-treated extract of strain 3/62 lost 75 % of its enzyme *B* activity within 30 min. under the same conditions. Purified enzyme *B* was even more sensitive than the cobamide-containing enzyme to treatment with cyanide; 50 % of the activity was lost in 30 min. at 22° and pH 8 with 0·07 mM-potassium cyanide, whereas 50 % inactivation of the cobamide-containing enzyme required 2·2 mM-potassium cyanide under the same conditions (Foster *et al.* 1961*a*).

Cobalamin content of Escherichia coli

The cobalamin content of thick suspensions of strains PA 15 and 121/176 was assayed with *Euglena gracilis*; in both cases the value obtained was 2·7 μmg./g. dry wt. of organisms when growth was in minimal medium supplemented with DL-methionine (0·5 mM) and glycine (10 mM); the value for strain PA 15 was unchanged by the omission of methionine from the medium. Direct total count of the organisms indicated that this value corresponded to less than half a molecule of cobalamin per organism.

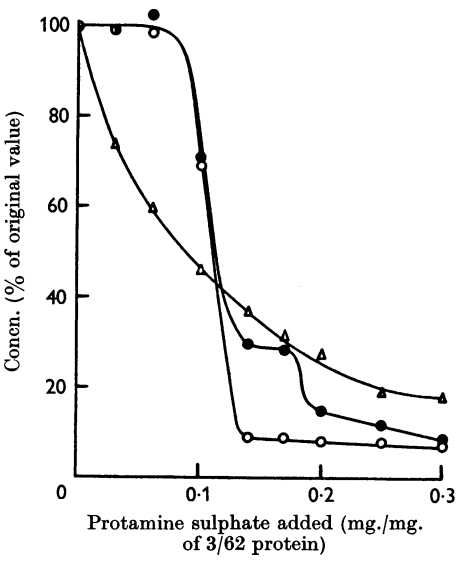


Fig. 6. Separation of enzyme *B* and the cobamide-containing enzyme. Protamine sulphate was added as shown to samples of extract of *E. coli* 3/62 grown with cobalamin. After standing for 30 min. at 0°, the mixtures were centrifuged and the supernatant fluids assayed for enzyme *B* + cobamide-containing enzyme (●), cobamide-containing enzyme (○) and protein (△).

DISCUSSION

For the sake of clarity in interpreting the results, tetrahydropteroyltriglutamate (H_4PtG_3) is taken to include the active factors present in the extract of heated *E. coli*; there is good evidence (Jones *et al.* 1961; Guest & Woods, 1962*a*) that one of these is the N^5-formyl derivative of H_4PtG_3.

E. coli 3/62 (derived from strain 518) is an auxotroph responding for growth only to methionine and not to homocysteine or cobalamin. As expected cell-free extracts do not convert homocysteine into methionine in the presence of substrates and co-factors effective with extracts of strains (e.g. PA 15) having no growth requirement for methionine. The enzyme (*A*) lacking in this mutant can be supplied by extracts of other strains of *E. coli*, including 121/176 and 113/3 which are auxotrophs with a growth response to either methionine or cobalamin. Such activation by enzyme *A* occurs only with H_4PtG_3, and not with H_4PtG unless cobalamin is also added; this is in accordance with previous results with strain PA 15, which is competent to synthesize methionine, where H_4PtG was an effective cofactor only when cobalamin was provided (Kisliuk & Woods, 1960; Guest *et al.* 1960). The activation of extract of strain 3/62 to convert homocysteine into methionine under standard conditions provided means of estimating enzyme *A*; it was thus possible to assay a single enzymic component of a complex system of enzymes without knowledge of the nature of that enzyme or its primary product. An assay of this kind would, of course, also detect an apoenzyme or any other enzyme in the test material able to fulfil the function of the enzyme under test; nevertheless this approach might be expected in general to give information about the individual reactions of a multi-enzyme sequence. A purification of enzyme *A* was achieved by using this assay method, but the final product may represent an apoenzyme rather than an intact holoenzyme.

The purification of enzyme *A* from strain 121/176, and of enzyme *B* from strain 3/62, rather than from strains competent to synthesize methionine, is of interest for three reasons. First, the crude extracts to be assayed are themselves unable to form methionine from homocysteine and serine, thus avoiding any uncertainty about subtraction of endogenous methionine synthesis. Secondly, each enzyme preparation is automatically freed of at least one enzyme related to it in metabolic function, e.g. enzyme *A* is free of enzyme *B*. Finally, the auxotrophic strains were a richer source of each

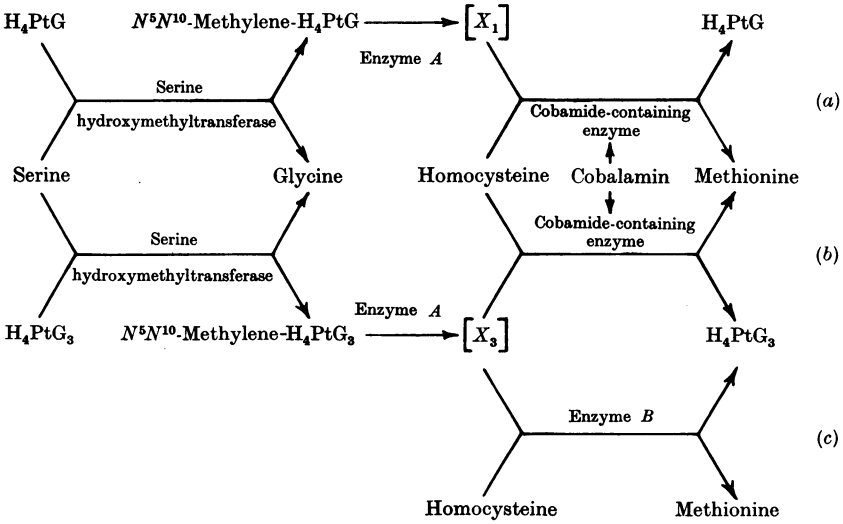
enzyme than prototrophic strains; this is probably a result (Foster, Rowbury & Woods, 1963) of harvesting the auxotrophs after prolonged growth in a medium with a poor supply of methionine, i.e. conditions where relief from repression might occur (Gorini & Maas, 1958).

Incubation of N^5N^{10}-methylene-H_4PtG_3 (representing the product of serine hydroxymethyltransferase action on serine) with enzyme *A*, either purified or present in a crude extract of strain 121/176, leads to the accumulation of a thermostable product (X_3); this gives methionine on subsequent incubation with homocysteine and either purified enzyme *B* or an extract of strain 3/62 grown with methionine (i.e. containing enzyme *B* but neither the cobamide-containing enzyme nor enzyme *A*). Similarly, N^5N^{10}-methylene-H_4PtG leads to an intermediate (X_1), but here subsequent conversion into methionine with homocysteine requires the cobamide-containing enzyme, provided as an extract of strain 3/62 grown with both methionine and cobalamin. The characterization of X_1 and X_3 as the N^5-methyl derivatives of H_4PtG and H_4PtG_3 respectively, together with the demonstration of the requirements for their synthesis by enzyme *A*, is described by Guest, Foster & Woods (1964*a*). The present results demonstrate clearly that the metabolic lesion in the auxotrophs responding to methionine alone occurs earlier in the biosynthetic sequence than the lesion in auxotrophs responding alternatively to methionine or to cobalamin.

As expected from previous studies in this Laboratory, methionine synthesis by an extract of strain 121/176, prepared from organisms grown with methionine, can be promoted more efficiently by the purified cobamide-containing enzyme than by cobalamin. Activation by the cobamide-containing enzyme occurs with either H_4PtG or H_4PtG_3 as cofactor and, unlike activation by cobalamin, is not blocked by the anilide analogue of cobalamin. However, the extract of strain 121/176 can also be activated to form methionine by extracts of other strains of *E. coli* (e.g. 3/62) even when each strain used had been grown without added cobalamin. Such activation cannot be via the cobamide-containing enzyme since the effect is found with H_4PtG_3 only; H_4PtG is inhibitory and its use as an effective cofactor still requires that cobalamin be added.

The requirements for methionine synthesis by mixtures of extracts of strain 3/62 and 121/176 are similar to those described for strain PA 15 (Guest *et al.* 1960) or strain 113/3 (Hatch *et al.* 1961). The functions of these cofactors have been investigated and are described and discussed by Guest *et al.* (1964*a*) and Guest, Friedman, Foster, Tejerina & Woods (1964*b*).

Taken as a whole, the present results, together with the demonstration of the formation of the cobamide-containing enzyme by strains PA 15, 3/62 and 121/176 (Guest & Woods, 1960), show that *E. coli* (or strains PA 15, 518 and W at least) can methylate homocysteine by two different enzymic mechanisms (Scheme 1). In the absence of added cobalamin, C-3 of serine is converted into the methyl group of methionine via serine hydroxymethyltransferase, enzyme *A* and enzyme *B* with H_4PtG_3 as cofactor (pathway *c*). When cobalamin is available, a second mechanism can operate by using the cobamide-containing enzyme as an alternative to enzyme *B* (pathway *b*). Although in



Scheme 1. Possible enzymic mechanisms for methionine synthesis from homocysteine and serine.

both mechanisms H_4PtG_3 can be used as cofactor, H_4PtG is effective in only the cobalamin-dependent mechanism (pathway *a*) and inhibits pathway *c*. Mutation to loss of enzyme *A* gives an auxotroph responding to methionine alone, but mutation to loss of enzyme *B* gives an auxotroph responding to either methionine or cobalamin.

That two pathways exist is also supported by earlier findings in this Laboratory. A requirement for cobalamin in methionine synthesis by extracts of *E. coli* has been found only when either (*a*) the extracts were derived from cobalamin auxotrophs (Guest *et al.* 1960), or (*b*) when H_4PtG rather than H_4PtG_3 was used as a cofactor with any of the strains tested (Kisliuk & Woods, 1960; Guest *et al.* 1960; Jones *et al.* 1961). Further, H_4PtG actually inhibits the function of H_4PtG_3, derivatives of which occur naturally. Finally, although cobalamin analogues inhibit the growth (on cobalamin) of cobalamin auxotrophs, they do not inhibit the growth of strains not requiring the vitamin (Guest, 1960).

The assay developed for enzyme *B*, analogous to that for enzyme *A*, enabled sufficient purification of the enzyme to show that it is distinct from the cobamide-containing enzyme (e.g. in terms of cobalamin content) even though the two enzymes act alternatively when H_4PtG_3 is the cofactor. Since all the known cobalamin auxotrophs of *E. coli* will also grow when supplied with methionine, it follows that cobalamin should not be present in any strain grown in its absence if the normal mechanism of methionine synthesis is independent of cobalamin. This was supported by the finding that strain PA 15 contains negligible amounts of growth-promoting activity for *Euglena gracilis* (even after treatment to release the vitamin as cyanocobalamin), and certainly no more than the cobalamin auxotroph grown with methionine. Thus it is concluded that cobalamin auxotrophs such as 121/176 or 113/3 have not, as has been generally supposed (e.g. Hatch *et al.* 1961), lost the ability to synthesize cobalamin, but have lost enzyme *B* and are therefore dependent on cobalamin being provided and the ability to synthesize the cobamide-containing enzyme. The synthesis of methionine via enzyme *B* can be considered the constitutive pathway; the cobalamin system represents an adaptive mechanism evoked by the provision of cobalamin.

SUMMARY

1. The formation of methionine from homocysteine has been studied by using mixtures of cell-free extracts of methionine auxotrophs of *Escherichia coli* blocked between homocysteine and methionine and giving growth responses (*a*) only with methionine or (*b*) with methionine or cobalamin. Methionine formation occurred only when extracts of type (*a*) and (*b*) auxotrophs were mixed, and it was deduced that each auxotroph lacks an enzyme present in the other.

2. Enzyme *A*, present in the methionine/cobalamin auxotroph, and also in methionine-independent strains of *E. coli*, has been purified 80-fold. It catalyses the formation of heat-stable intermediates, X_1 and X_3, from N^5N^{10}-methylenetetrahydropteroyl-monoglutamate and -triglutamate respectively.

3. Enzyme *B* catalyses a reaction between X_3 (though not X_1) and homocysteine, yielding methionine. It has been purified 15-fold from extracts of an auxotroph responding to methionine only and is also present in strains competent to synthesize methionine.

4. Methionine formation from homocysteine and X_1 requires a cobamide-containing enzyme present in methionine/cobalamin auxotrophs and other strains of *E. coli* only when they are grown in the presence of cobalamin. This enzyme can also use X_3 as substrate.

5. Enzyme *B* is free from the cobamide-containing enzyme, contains a negligible amount of cobalamin and can be separated from the cobamide-containing enzyme when both are present in the same extract.

6. A strain of *E. coli* fully competent to synthesize methionine when harvested from a medium devoid of cobalamin was found to contain a negligible amount of cobalamin.

7. It is concluded that methionine formation normally occurs through the successive action of enzymes *A* and *B*, is independent of cobalamin, but requires specifically tetrahydropteroyltriglutamate as cofactor. Growth in the presence of cobalamin evokes a second mechanism, obligatory for methionine/cobalamin auxotrophs, that depends on a cobamide-containing enzyme though either tetrahydropteroyl-monoglutamate or -triglutamate serves as cofactor.

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Methyl Derivatives of Folic Acid as Intermediates in the Methylation of Homocysteine by *Escherichia coli*

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The term folate is used in this paper in reference to both simple and conjugated members of the folic acid family of vitamins; tetrahydrofolate thus includes both 5,6,7,8-tetrahydropteroyl-monoglutamate and -triglutamate.*

The general background of the present work was fully reviewed in the preceding paper (Foster, Tejerina, Guest & Woods, 1964), in which was also presented experimental evidence that there are alternative pathways for the conversion of homocysteine into methionine by strains of *Escherichia coli*. These differ in the mechanism by which the C_1 unit is transferred (with concomitant reduction) from N^5N^{10}-methylenetetrahydrofolate to homocysteine (Scheme 1 in Foster *et al.* 1964). In one case only N^5N^{10}-methylene-H_4PtG_3 could be used as substrate; it was converted by an enzyme *A* into an unidentified intermediate (X_3) that reacted with homocysteine in the presence of an enzyme *B*; cobalamin derivatives were not required at either step. The alternative mechanism was dependent on a cobamide-containing enzyme, clearly distinguishable from enzyme *B*. In this case the N^5N^{10}-methylene derivative of either H_4PtG or H_4PtG_3 was an effective substrate and they were converted by enzyme *A* into intermediates X_1 and X_3 respectively; both X_1 and X_3 yielded methionine when incubated with homocysteine and an enzymic extract possessing the cobamide-containing enzyme but not enzyme *B*.

The object of the present work was to identify the intermediates X_1 and X_3 and to study in more detail the mechanism of their formation by enzyme *A*. *E. coli* 121/176 (an auxotroph giving a growth response with either methionine or cobalamin) was used as a specific source of this enzyme since it lacks enzyme *B* and possesses no cobamide-containing enzyme when grown with methionine (Table 1).

The technique used for the identification of X_1 (and consequently of X_3) was aided by the publica-

* Abbreviations for members of the folic acid group, based on PtG for pteroylglutamate: H_4PtG, tetrahydropteroylmonoglutamate; H_4PtG_3, tetrahydropteroyltriglutamate; H_2PtG, dihydropteroylglutamate; derivatives of these compounds are indicated as, e.g., N^5N^{10}-methylene-H_4PtG, N^5-methyl-H_4PtG_3.