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Cobalamin and the Synthesis of Methionine by Ultrasonic Extracts of *Escherichia coli*

BY J. R. GUEST, C. W. HELLEINER,* M. J. CROSS† AND D. D. WOODS
Microbiology Unit, Department of Biochemistry, University of Oxford

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The synthesis of methionine from homocysteine and serine by *Escherichia coli* has been studied in these laboratories with suspensions of intact organisms (Gibson & Woods, 1960) and with enzymic extracts (Cross & Woods, 1954; Szulmajster & Woods, 1960; Kisliuk & Woods, 1960). With whole organisms strong presumptive evidence was obtained that some form of folic acid was required as coenzyme for the C_1-unit transfer and this was confirmed by the studies with enzyme preparations, though tetrahydropteroylglutamic acid and other forms of folic acid tried were not effective sources of the folic acid coenzyme (Cross & Woods, 1954; Szulmajster & Woods, 1955, 1960). Extracts of acetone-dried organisms were, however, activated by tetrahydropteroylglutamic acid provided that the organisms had been harvested from a medium containing cobalamin (Kisliuk & Woods, 1957, 1960).

Using an auxotrophic strain of *E. coli* requiring methionine or cobalamin for growth, Gibson & Woods (1952, 1960) found that washed suspensions of organisms harvested from a medium devoid of cobalamin did not synthesize methionine unless the vitamin was added. The formation of methionine by suspensions of a number of strains of *E. coli* not requiring cobalamin for growth was also increased markedly by the vitamin, the largest effect (over fivefold) being obtained with an auxotrophic strain (PA 15) with a growth requirement for serine or glycine. Most of the work with enzymic extracts quoted above was done with extracts of acetone-dried powders of this strain and no direct effect of cobalamin on the activity of the extracts was found.

In the present work the primary enzyme preparations were made by ultrasonic vibration instead of by drying with acetone. An effect of cobalamin was at once apparent under all conditions with a mutant requiring cobalamin or methionine for growth (and grown with methionine). Cobalamin now also activated methionine synthesis under certain conditions with strain PA 15; it enabled tetrahydropteroylglutamate to serve as a source of the folic acid coenzyme also required in the reaction. Brief accounts of part of this work have already been published (Helleiner & Woods, 1956; Helleiner, Kisliuk & Woods, 1957; Woods, 1958). Towards the end of this work a brief account appeared of a cobalamin-containing enzyme, forming part of the complex of enzymes (in a cobalamin auxotroph of *E. coli*), which catalyses the transfer of C-3 of serine to homocysteine (Hatch, Takeyama & Buchanan, 1959).

There have been several investigations of the formation of methionine from homocysteine by enzymic extracts of chicken, sheep and pig liver with serine or formaldehyde as donor of the C_1 unit (Doctor, Patton & Awapara, 1957; Nakao & Greenberg, 1958; Stevens & Sakami, 1959). No effect of cobalamin was described; tetrahydropteroylglutamic acid was an effective source of folic acid with sheep- and pig-liver preparations and pteroylglutamic acid itself was active with the enzyme from chicken liver.

* Post-doctoral Fellow, National Research Council of Canada at the time of this work.

† Guinness Research Fellow in Microbiological Biochemistry at the time of this work.

MATERIALS AND METHODS

Organisms and enzymic extracts

Organisms. Stock cultures of all organisms were maintained on slopes of tryptic meat–agar, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°. The greater part of the work was done with two auxotrophic strains of *E. coli*. Strain 121/176, which requires cobalamin or methionine for growth, was as described by Davis & Mingioli (1950) and was obtained from Dr B. D. Davis. Strain PA15 requires glycine or serine and was originally obtained from Dr Barbara Wright; it has been used in previous studies of methionine synthesis in these laboratories (Gibson & Woods, 1960; Szulmajster & Woods, 1960; Kisliuk & Woods, 1960). Other strains used in a few experiments were 113/3, another cobalamin or methionine auxotroph (Davis & Mingioli, 1950), and two prototrophs, W (the parent strain of 121/176 and 113/3) and 518 (ATCC no. 9723).

Media and growth. The basal medium for bulk growth contained (g./l.): K_2HPO_4, 19·2; KH_2PO_4, 8·8; $(NH_4)_2SO_4$, 2·0; NH_4Cl, 5·0; $MgSO_4,7H_2O$, 0·2; glucose, 4·0; lactic acid, 20·0. After addition of Fe^{2+} ions (final concn. 8 μM) from a solution of $FeSO_4,(NH_4)_2SO_4,6H_2O$ in twice its molar concentration of sodium citrate, the pH was brought to 7 with 10N-NaOH. The medium was supplemented with DL-methionine (0·5 mM) for strains 121/176 and 113/3, and with glycine (10 mM) for strain PA15. After addition of 3% (w/v) of agar and sterilization (121°; 10 min.) the medium was dispensed either in Roux bottles (100 ml.) or enamelled trays (as described by Szulmajster & Woods, 1960) (600 ml.).

In a few experiments cobalamin replaced methionine as growth factor for strain 121/176 or was added to the complete medium for strain PA15; it was added as a sterile solution to the bulk medium.

The inoculum for a Roux bottle was about 1·5 mg. dry wt. of organisms from a tryptic meat–agar slope harvested after incubation for 16 hr. at 37°. Each tray received about 3 mg. dry wt. of organisms from a 16 hr. culture (37°) on minimal medium (Davis & Mingioli, 1950) supplemented with any required growth factor. Both trays and Roux bottles were incubated for 44 hr. at 37°. The organisms were harvested, washed twice in the centrifuge with either water or 0·85% NaCl solution (140 ml./tray; 45 ml./Roux bottle), and finally suspended in either 0·2M-phosphate buffer, pH 7·3, or 0·05M-phosphate buffer, pH 7·8, according to the reaction mixture used for studying methionine synthesis (see below).

Preparation of enzymic extracts. Washed suspensions (10–25 ml.) containing 75–100 mg. dry wt. of organisms/ml. were subjected to ultrasonic vibration (25 kcyc./sec., 4 min., 600 w) in a cooled, stainless-steel, conical flask, to the base of which the magneto-strictor was brazed. The ultrasonic generator was type E 7590 B of Mullard Ltd., London, W.C. 1. After centrifuging at 0° (25 000 *g*/10 min.) the supernatant fluid was dialysed either against running tap water overnight or against two changes of 2 l. quantities of 0·05M-phosphate buffer, pH 7·8, at 3°. The dialysed preparation was centrifuged to remove precipitated protein and the protein content of the supernatant (which was used as enzyme source) was estimated spectrophotometrically according to Layne (1957).

In some experiments the dialysed extract was treated with Dowex-1 resin; the procedure was that of Kisliuk & Woods (1960), except that the resin (chloride form) was X8, 100–200 mesh.

The enzyme preparations were normally used within 2 days of preparation since that from strain 121/176 progressively lost activity (50% in 3 weeks) when stored at either 2° or −15°; the preparation from strain PA15 was more stable (15% loss in 3 weeks).

Extract of heated organisms. Washed suspensions (170 mg. dry wt./ml.) of the strain under test were heated for 3 min. at 100°, cooled and centrifuged. The supernatant will be referred to as extract of heated *E. coli*; it contained 0·15 μmole of methionine/ml.

Assessment of mass of organisms. This was estimated photometrically as described by Gibson & Woods (1960).

Test for methionine synthesis

Reaction mixtures. The solutions used for testing methionine synthesis by ultrasonic extracts were based on the work of Cross & Woods (1954) and Kisliuk & Woods (1960). Basal reaction mixture *A* was used for most of the work with *E. coli* 121/176; it contained, in 2 ml. of potassium phosphate buffer (0·05M, pH 7·3): DL-serine, 20 μmoles; DL-homocysteine, 20 μmoles; glucose, 20 μmoles; $MgCl_2$, 10 μmoles; adenosine triphosphate (ATP), 4 μmoles; diphosphopyridine nucleotide (DPN), 0·4 μmole. Basal reaction mixture *B* was used mainly with *E. coli* PA15 but also in the later work with strain 121/176. It contained [in 2 ml. of potassium phosphate buffer (0·125M, pH 7·8)]: L-serine, 10 μmoles; DL-homocysteine, 10 μmoles; glucose, 20 μmoles; $MgCl_2$, 10 μmoles; ATP, 10 μmoles; DPN, 1 μmole; pyridoxal phosphate, 0·5 μmole. In experiments with the formaldehyde derivative of tetrahydropteroylglutamic acid as the C_1-unit donor the amount of DL-homocysteine was decreased to 5 μmoles and pyridoxal phosphate was omitted (Kisliuk & Woods, 1960).

When present, extract of heated *E. coli* was added to both reaction mixtures in an amount equivalent to 34 mg. dry wt. of the original organisms. The amount of enzymic extract used as a routine contained 10 mg. of protein.

The complete reaction mixture was incubated at 37° for 4–6 hr. and normally under an atmosphere of H_2, though static incubation in air was sometimes used when tetrahydropteroylglutamic acid was not present. After heating (100°, 3 min.) the reaction mixture was centrifuged to remove precipitated protein and the supernatant fluid used for the assay of methionine.

Estimation of methionine. Microbiological assay with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 was carried out as described by Gibson & Woods (1960) with DL-methionine as standard; the organism responds only to the L-isomer. Additional information on the specificity of the assay is given by Kisliuk & Woods (1960).

Expression of results. The amount of L-methionine formed is given throughout in terms of μm-moles/mg. of protein of the enzyme preparation in the time incubated; results are not given per unit of time since in some cases there was a lag in methionine formation during the first hour of incubation.

A control in which a reaction mixture containing all components was heated at zero time was included in each

experiment; the value obtained (about 4 μm-moles of L-methionine/mg. of protein) was deducted from the experimental and other control values.

Chemicals

Cobalamin and derivatives. The source of cobalamin was Anacobin (British Drug Houses Ltd., Poole, Dorset). It was about 90% pure when assayed spectrophotometrically with the extinction coefficient quoted by Brink *et al.* (1949).

Pseudovitamin B_{12}, factors A, F and III and the 5-methylbenziminazole, 5:6-dichlorobenziminazole and 2:6-diaminopurine analogues of cobalamin were provided by Dr J. E. Ford; factor B was given by Dr E. Lester Smith. The chemical nature and biological properties of these members of the vitamin B_{12} group are summarized by Kon & Pawelkiewicz (1958).

The specimen of 5:6-dimethylbenzimidazolylcobamide coenzyme was given by Dr H. A. Barker. In experiments with this compound precautions were taken at all stages to exclude light.

Irradiated specimens of both cobalamin and 5:6-dimethylbenzimidazole B_{12}-coenzyme were prepared by exposing 0·3 ml. of a 10 μM-solution in a silica cell for 50 min. at 6 ft. from an 80 w white fluorescent light. This was sufficient to cause the changes in absorption spectrum reported by Barker, Weissbach & Smyth (1958).

Folic acid derivatives. Tetrahydropteroylglutamic acid (tetrahydro-PtGA) and its formaldehyde derivative were prepared and standardized by methods quoted by Kisliuk & Woods (1960). The formaldehyde derivative (which will be referred to as tetrahydro-PtGA–CH_2O) is believed to be N^5N^{10}-methylenetetrahydropteroylglutamic acid (Kisliuk, 1957; Blakley, 1959).

Other chemicals. Adenosine triphosphate, diphosphopyridine nucleotide (and its reduced form, $DPNH_2$) and triphosphopyridine nucleotide (TPN) were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. The DL-homocysteine was from either Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. or Mann Research Laboratories Inc., New York, N.Y., U.S.A.

RESULTS

E. coli 121/176 is an auxotroph requiring either cobalamin or methionine for growth. In order to obtain organisms and enzyme preparations which could be expected to be relatively deficient in cobalamin it was grown with methionine as supplement; concentrations only just optimum for growth were used since higher ones were likely to repress the formation of the enzyme systems under investigation (Wijesundera & Woods, 1960). Strain PA 15 does not require cobalamin for growth, but synthesis of methionine by suspensions of whole organisms is increased several-fold by the vitamin (Gibson & Woods, 1960).

The effect of cobalamin on the synthesis of methionine from homocysteine by ultrasonic extracts of both strains was examined under three sets of conditions with regard to the nature of the

Table 1. *Effect of cobalamin when extract of heated* Escherichia coli *is the source of folic acid coenzyme*

Strain 121/176: reaction mixture *A* plus enzymic extract, extract of heated *E. coli* and cobalamin (14 $\mu\mu$moles). Incubated for 4 hr.

Strain PA 15: reaction mixture *B* plus Dowex-treated enzymic extract and extract of heated *E. coli*. Cobalamin, 70 $\mu\mu$moles, when present. Incubated for 4 hr.

Modifications to reaction mixtures		L-Methionine formed (μm-moles/mg. of protein)	
Omissions	Additions	Strain 121/176	Strain PA 15
None	—	24	76
None	Cobalamin	—	75
Cobalamin	—	6	—

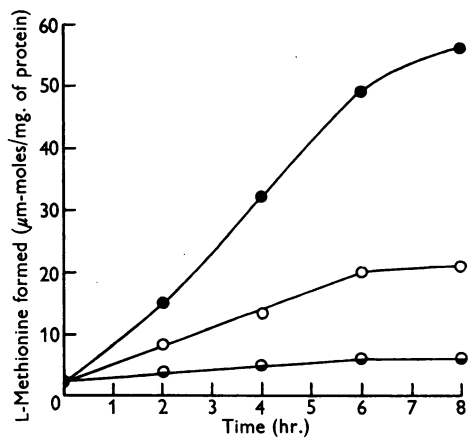

Fig. 1. Effect of cobalamin on the rate of formation of methionine by enzymic extracts of *E. coli* 121/176 when extract of heated organisms is the source of folic acid coenzyme. Reaction mixture *A* plus enzymic extract, extract of treated *E. coli* and cobalamin (when present), 14 $\mu\mu$moles. ●, Complete system; ○, no cobalamin; ◒, no DL-homocysteine.

Table 2. *Effect of the concentration of cobalamin on methionine synthesis by extracts of* Escherichia coli 121/176

Reaction mixture *B* plus enzymic extract and either extract of heated *E. coli* or tetrahydro-PtGA (1 μmole). Incubated for 4 hr.

Cobalamin added ($\mu\mu$moles)	L-Methionine formed (μm-moles/mg. of protein)	
	Extract of heated *E. coli*	Tetrahydro-PtGA
0	6	3
14	18	25
28	25	40
70	40	62
350	44	88

donor of the C_1 unit and the nature of the source of the coenzyme form of folic acid (CoF): (*a*) serine with extract of heated *E. coli*, (*b*) serine with tetrahydropteroylglutamic acid, and (*c*) the formaldehyde derivative of tetrahydro-PtGA (tetrahydro-PtGA–CH_2O) as both donor and CoF source. For reasons discussed by Kisliuk & Woods (1960) it is not possible to use free formaldehyde as a donor of C_1 units when homocysteine is present.

The activity of the enzymic extracts of both strains varied overall about threefold from one preparation to another. This may be due to a number of enzymes with varying stability being required for the overall reaction: the work of Emerson (1950) with mutants of *Neurospora* suggests that the reaction may be complex.

Effect of cobalamin

Serine with extract of heated E. coli. Under these conditions methionine was synthesized by extracts of strain PA15 in the absence of cobalamin and its addition did not improve the yield. On the other hand, synthesis by strain 121/176 was increased about fourfold by cobalamin (Table 1); after a slight initial lag methionine formation was proportional to time for 4 to 6 hr. and was negligible in the absence of homocysteine (Fig. 1). The effect of cobalamin concentration (of which an example is given in Table 2) was variable in the sense that the higher concentrations did not always give further increases in methionine synthesis; with some enzyme preparations, however, the increases were considerably greater than those shown in Table 2.

Serine with tetrahydropteroylglutamic acid. Cobalamin was required for methionine synthesis not only by the cobalamin-deficient enzymic extracts of strain 121/176 but also by the extracts of strain PA15 (Table 3 and Fig. 2). The rate of methionine synthesis with strain PA15 (Fig. 2) shows an initial lag of about 1 hr. before becoming almost linear up to 4 hr.; the lag period was usually decreased if a much larger quantity of cobalamin (about 1 μm-mole) was used, although the maximum rate of methionine formation was not necessarily increased.

The effect of concentration of cobalamin with tetrahydro-PtGA present is shown in Table 2 for

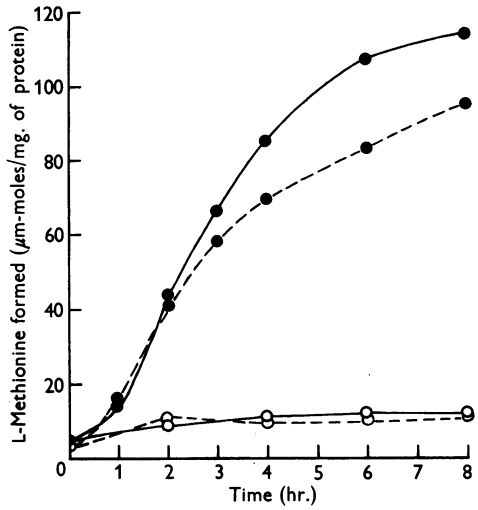

Fig. 2. Effect of cobalamin on the rate of formation of methionine by enzymic extracts of *E. coli* PA15 in the presence of tetrahydropteroylglutamic acid (—) or its formaldehyde derivative (- - -). —, Reaction mixture *B* with enzymic extract and tetrahydro-PtGA (1 μmole). - - -, Reaction mixture *B* (without L-serine and pyridoxal phosphate and with DL-homocysteine decreased to 5 μ-moles) plus enzymic extract and tetrahydro-PtGA–CH_2O (3·3 μmoles). Cobalamin: present (70 $\mu\mu$moles) (●) or absent (○).

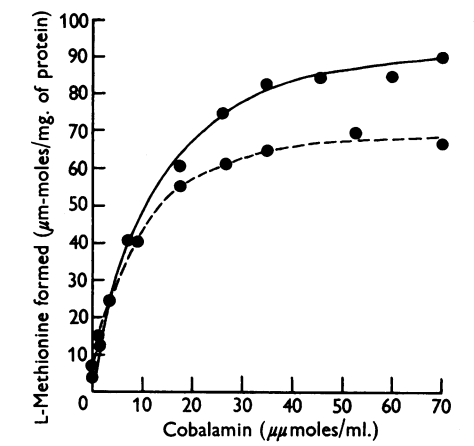

Fig. 3. Effect of the concentration of cobalamin on the synthesis of methionine by enzymic extracts of *E. coli* PA15 in the presence of tetrahydropteroylglutamic acid or its formaldehyde derivative. Conditions for — (tetrahydro-PtGA present) and - - - (tetrahydro-PtGA–CH_2O present) were as described in Fig. 2. Incubated for 4 hr.

Table 3. *Requirement for cobalamin when tetrahydropteroylglutamic acid is the source of folic acid coenzyme*

Reaction mixture *B* plus Dowex-treated enzymic extracts and either extract of heated *E. coli* or tetrahydro-PtGA (1 μmole). Incubated for 4 hr. Cobalamin: +, present (70 $\mu\mu$moles); −, absent.

Source of CoF	Cobalamin	L-Methionine formed (μm-moles/mg. of protein) Strain 121/176	Strain PA15
Extract of heated *E. coli*	+	40	55
Tetrahydro-PtGA	−	3	5
Tetrahydro-PtGA	+	62	80

Table 4. *Requirement for cobalamin when the formaldehyde derivative of tetrahydropteroylglutamic acid replaces both folic acid and serine*

Strain 121/176: reaction mixture *A* (with DL-serine omitted) plus enzymic extract. Cobalamin (14 μμmoles), tetrahydro-PtGA–CH_2O (3·6 μmoles), extract of heated *E. coli* (as in Materials and Methods section) and DL-serine (20 μmoles when present). Incubated for 4 hr.

Strain PA15: reaction mixture *B* (with L-serine omitted) plus enzymic extract. Cobalamin (14 μμmoles), tetrahydro-PtGA–CH_2O (3·3 μmoles), extract of heated *E. coli* (as in Materials and Methods section) and L-serine (10 μmoles when present). Incubated for 4 hr.

Source of C_1 unit	Source of CoF	Cobalamin	L-Methionine formed (μm-moles/mg. of protein) Strain 121/176	Strain PA15
Serine	Extract of heated *E. coli*	Present	24	103‡
Tetrahydro-PtGA–CH_2O		Absent	6*	4
Tetrahydro-PtGA–CH_2O		Present	38†	40

* In four other experiments results ranged from 4 to 12.
† In four other experiments results ranged from 22 to 92.
‡ 101 when cobalamin was absent.

enzymic extracts of strain 121/176 and in Fig. 3 for those from strain PA15. The results were again variable in the sense described in the previous section for methionine synthesis in the presence of extract of heated *E. coli*.

Formaldehyde derivative of tetrahydropteroylglutamic acid. When tetrahydro-PtGA–CH_2O was used as source both of C_1 units and of folic acid coenzyme the synthesis of methionine by enzymic extracts of both strains again required cobalamin (Table 4, Fig. 2). The rate of methionine synthesis with strain PA15 was only slightly less than that when serine was donor and tetrahydro-PtGA the source of CoF (Fig. 2). The effect of increasing cobalamin concentrations was again variable with different enzyme preparations, but with the same extract similar results were obtained (Fig. 3) to those for the serine–tetrahydro-PtGA system.

Analogues and derivatives of cobalamin. These tests were carried out only with enzymic extracts of strain PA15 with serine as C_1-unit donor and tetrahydro-PtGA as source of folic acid coenzyme.

Coenzyme forms of vitamin B_{12} active in the metabolism of glutamate by *Clostridium tetanomorphum* have recently been described (Barker *et al.* 1958; Weissbach, Toohey & Barker, 1959). The 5:6-dimethylbenzimidazolylcobamide coenzyme was two to three times as active as cobalamin in the present test system (Fig. 4); the concentrations for half-maximal activation were 0·015 and 0·038 μM for the coenzyme and cobalamin respectively. Previous irradiation had no effect on the activity of cobalamin, but greatly decreased that of the coenzyme (Fig. 4). With the latter the effect of irradiation was apparently more marked at higher concentrations than at lower, suggesting the possibility that an inhibitor was formed.

A number of other compounds in which the

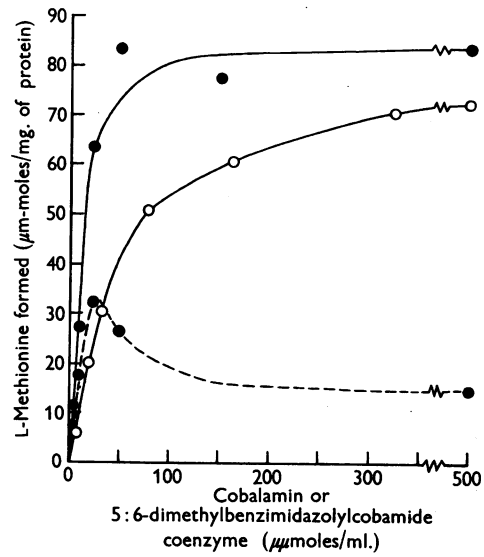

Fig. 4. Comparative activity of cobalamin and vitamin B_{12}-coenzyme. Cobalamin (with or without irradiation), ○; 5:6-dimethylbenzimidazole B_{12}-coenzyme (with, - - -, or without, —, irradiation), ●. Reaction mixture *B* plus tetrahydro-PtGA (1 μmole) and Dowex-treated enzymic extract of strain PA15. Irradiation of cobalamin and coenzyme was as described in the Materials and Methods section. Incubated for 3 hr.

nature of the base of the nucleotide residue differed from that in cobalamin was tested (Table 5); none had significantly higher activity than cobalamin itself at the concentrations tested. The substance with lowest activity was factor B (which has no nucleotide residue), but the further addition of 5:6-dimethylbenziminazole (itself inactive) resulted in activity equivalent to one-third of that of cobalamin. There was no quantitative correlation

with the relative growth-promoting activity of these compounds for the cobalamin auxotroph *E. coli* 113/3 as given by Kon & Pawelkiewicz (1958).

Presence of cobalamin during growth. In all the experiments so far described enzyme preparations were made from organisms grown in the absence of added cobalamin. Strain 121/176 requires either cobalamin or methionine for growth: when cobalamin at relatively low concentrations was added to the original growth medium an effect of cobalamin on methionine synthesis by the enzyme preparations was still obtained (Table 6). The effect was even significant at a concentration of cobalamin in the growth medium in excess of that required for optimum growth of the organism (0·7 $\mu\mu$mole/ml.). Results were similar when extract of heated *E. coli* served as source of folic acid coenzyme. Strain PA 15 does not require cobalamin for growth, but its effect with enzymic extracts was not abolished unless the concentration in the growth medium exceeded 1·4 $\mu\mu$mole/ml. (Table 6).

It was also observed that increasing the concentration of cobalamin in the growth medium progressively increased the overall ability of the enzymic extracts of both organisms to synthesize methionine (Table 6); the rate of formation, however, decreased sharply after 2 hr. incubation.

Other strains of Escherichia coli. An effect of cobalamin with enzymic extracts of three other strains of *E. coli* was shown under appropriate conditions; all three systems were tested with regard to the nature of the donor of the C_1 unit and the source of folic acid coenzyme (Table 7). Strain 113/3 (like strain 121/176) is an auxotroph requiring cobalamin or methionine for growth; methionine synthesis by enzymic extracts was increased (as with strain 121/176) by cobalamin under all three conditions, though the effect was more marked when tetrahydro-PtGA rather than the extract of heated *E. coli* was the source of folic acid coenzyme. With the two prototrophic strains (518 and W) there was (as with strain PA 15) no effect of cobalamin when extract of heated *E. coli* was used; however, with tetrahydro-PtGA present synthesis of methionine by enzymic extracts, though small, was markedly increased by the vitamin (Table 7).

Table 5. *Ability of analogues to replace cobalamin*

Reaction mixture *B* plus tetrahydro-PtGA (1 μmole) and enzymic extract of strain PA15. Incubated for 4 hr. Factor B, etc., indicate trivial names of the whole compound.

Nature of the base of the nucleotide residue	Amount of test compound ($\mu\mu$moles)	
	70	700
	Activity compared with cobalamin (%)	
No nucleotide present (factor B)	7	7
5-Methylbenzimidazole	5	45
Adenine (pseudovitamin B_{12})	30	100
2:6-Diaminopurine	55	80
5-Hydroxybenzimidazole (factor III)	85	90
2-Methyladenine (factor A)	100	95
5:6-Dichlorobenzimidazole	100	100
2-Methylmercaptoadenine	125	100

Effect of folic acid

The presence of some form of folic acid was necessary for appreciable synthesis of methionine by extracts of both organisms (strains 121/176 and PA 15) under all test conditions investigated (Table 8); cobalamin did not affect the requirement. When tetrahydro-PtGA–CH_2O was used as donor of C_1 units the tetrahydro-PtGA residue was essential; replacement of tetrahydro-PtGA–CH_2O by the molar equivalent of formaldehyde decreased formation of methionine to low values (Table 8).

The effect of folic acid was studied in more detail with Dowex-treated extracts of strain PA 15 with

Table 6. *Effect of growth in the presence of cobalamin on its subsequent activity with enzymic extracts*

Methionine was omitted from the growth medium for strain 121/176 except when no cobalamin was added. Reaction mixture *B* with enzymic extract of either strain 121/176 or strain PA15 and tetrahydro-PtGA (1 μmole). Cobalamin, 0·7 μm-mole when present. Incubated for 2 hr.

Cobalamin in growth medium ($\mu\mu$moles/ml.)	L-Methionine formed (μm-moles/mg. of protein)			
	Strain 121/176		Strain PA15	
	With cobalamin	Without cobalamin	With cobalamin	Without cobalamin
0	6*	67*	2	44
0·05	18	69	—	—
0·28	35	75	8	42
1·4	71	107	26	58
3·5	102	124	66	72
7·0	125	126	85	86
21·0	125	129	107	107

* Methionine was added to the growth medium.

serine as donor of C_1 units and tetrahydro-PtGA as source of folic acid (cobalamin present); the optimum concentration was about 0·5 μmole/ml. of reaction mixture (Fig. 5). The ability of extract of heated *E. coli* to promote synthesis of methionine in the absence of cobalamin was almost completely inhibited by tetrahydro-PtGA at concentrations as low as 0·1 μmole/ml. (Fig. 5). The same result was obtained with tetrahydro-PtGA freshly prepared from pteroylglutamic acid which had been purified by chromatography (Sakami & Knowles, 1959).

Effect of other factors

Substrates. Omission of either serine or homocysteine decreased synthesis of methionine to low values in all cases tested (Table 9); it is clear that the enzyme preparations used contained little of these amino acids. Serine could not be replaced as donor of C_1 units either by glycine (Table 9) or its formimino derivative; *N*-formylglycine and formate were also inactive with the one strain tested (PA15). DL-Phosphoryl-*O*-serine had only 75% of

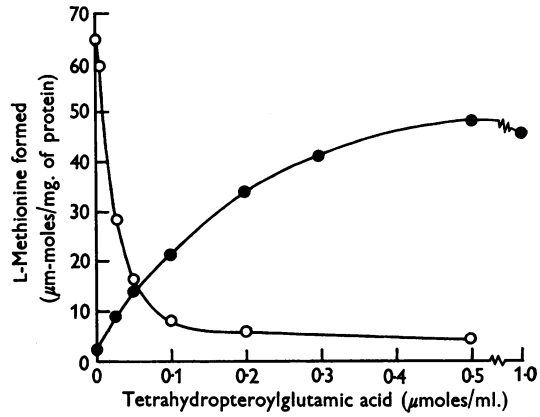

Fig. 5. Effect of the concentration of tetrahydropteroylglutamic acid on the synthesis of methionine by enzymic extracts of *E. coli* PA15 in the presence of either cobalamin or extract of heated *E. coli*. Reaction mixture *B* with Dowex-treated enzymic extract supplemented with either 70 μμmoles of cobalamin (●) or with extract of heated *E. coli* (○). Incubated for 4 hr.

Table 7. *Effect of cobalamin on the synthesis of methionine by extracts of other strains of* Escherichia coli

Reaction mixture *A* plus enzymic extracts and with DL-serine omitted if tetrahydro-PtGA–CH_2O was present. Cobalamin (14 μμmoles), tetrahydro-PtGA (1 μmole) and tetrahydro-PtGA–CH_2O (3·6 μmoles) when present; extract of heated *E. coli* was used as described in the Materials and Methods section. Incubated for 4 hr. Cobalamin: +, present; −, absent.

		L-Methionine formed (μm-moles/mg. of protein)					
C_1-unit donor ...		Serine		Serine		Tetrahydro-PtGA–CH_2O	
Source of CoF ...		Extract of heated *E. coli*		Tetrahydro-PtGA			
Cobalamin ...		−	+	−	+	−	+
Strain of *E. coli*	113/3	30	39	5	14	9	31
	518	16	16	—	—	2	6
	W	26	27	4	20	5	8

Table 8. *Effect of folic acid on methionine synthesis by enzymic extracts of both strains of* Escherichia coli

Strain 121/176: reaction mixture *A* (DL-serine omitted when tetrahydro-PtGA–CH_2O was present) plus enzymic extract and cobalamin (14 μμmoles).

Strain PA15: reaction mixture *B* (L-serine omitted when tetrahydro-PtGA–CH_2O was present) plus Dowex-treated enzymic extract and cobalamin (70 μμmoles). Other substances, when present: tetrahydro-PtGA (1 μmole), tetrahydro-PtGA–CH_2O (3·3 μmoles) and extract of heated *E. coli* (as in Materials and Methods section). Incubated for 4 hr.

Strain of *E. coli*	Conditions for methionine synthesis		L-Methionine formed (μm-moles/mg. of protein)	
	C_1 donor	Source of folic acid	With folic acid	Without folic acid
121/176	Serine	Extract of heated *E. coli*	5	24
PA15	Serine	Extract of heated *E. coli*	4	76
PA15	Serine	Tetrahydro-PtGA	7	70
121/176	Tetrahydro-PtGA–CH_2O		6*	38
PA15	Tetrahydro-PtGA–CH_2O		4*	68

* For these experiments tetrahydro-PtGA–CH_2O was omitted and formaldehyde (3·3 μmoles) added.

Table 9. *Effect of other components of the reaction mixture on methionine synthesis by both strains under various conditions*

Conditions were as described for Table 8. Other substances when present: TPN (1 μmole), $DPNH_2$ (10 μmoles), extract of heated *E. coli* (as in Materials and Methods section) and glycine (10 μmoles). Incubated for 4 hr.

	L-Methionine formed (μm-moles/mg. of protein)				
Nature of C_1 donor	Serine		Serine		
Source of CoF	Extract of heated *E. coli*		Tetrahydro-PtGA	Tetrahydro-PtGA–CH_2O	
Strain of *E. coli*	121/176	PA15	PA15	PA15	121/176
Omission from reaction mixture					
None	24	76	70	68	38
ATP + Mg^{2+} ion	14	15	12	15	13
DPN	24	68	10	11	7
DPN (TPN added)	—	25	11	17	—
None (TPN added)	—	61	75	68	—
Glucose	—	39	10	15	11
Glucose + DPN	—	—	5	—	—
Glucose + DPN ($DPNH_2$ added)	—	—	63	—	—
DL-Homocysteine	3	4	5	8	—
DL- or L-Serine	4	8	7	—	—
DL- or L-Serine (glycine added)	5	11	—	—	—

the activity of DL-serine with strain 121/176; it may be hydrolysed before use. The tests with alternative potential C_1 donors were done with extract of heated *E. coli* as source of folic acid in case other and unknown cofactors were required.

Diphosphopyridine nucleotide. The requirement for DPN, which was clear with the other test systems, could not be shown with extract of heated *E. coli* present (Table 9); it presumably contains sufficient DPN. In the three conditions tested with strain PA15, TPN did not replace DPN and was somewhat inhibitory when extract of heated *E. coli* was the source of folic acid coenzyme (Table 9).

Both glucose and DPN could be effectively replaced by the single addition of $DPNH_2$ at high concentrations (Table 9; strain PA15, serine–tetrahydro-PtGA system); furthermore, synthesis of methionine reached about half the maximum even when ATP was omitted as well.

Adenosine triphosphate and magnesium ions. The effect of omitting these substances from the reaction mixture was more marked with strain PA15 than with strain 121/176, possibly due to the higher level of synthesis of methionine by the former strain.

Enzyme concentration. In the one case tested (strain PA15 with the serine–tetrahydro-PtGA system) synthesis of methionine increased linearly with concentration of enzymic extract up to the limit tested (10 mg. of protein/ml.).

Presence of methionine during growth. Wijesundera & Woods (1960) observed that growth in the presence of the amino acid markedly decreased its subsequent synthesis from homocysteine and serine by suspensions of a number of strains of *E. coli*. Increase of the concentration of methionine in the growth medium of strain 121/176 to mM, or addition of this concentration of the amino acid to the medium for strain PA15, resulted in a decrease in the activity of the enzymic extracts to about a quarter.

DISCUSSION

Effect of cobalamin. Kisliuk & Woods (1957, 1960), working with extracts of acetone-dried powders of *E. coli* PA15, found that tetrahydro-PtGA was only used in the transfer of C_1 units to homocysteine provided that the organism had been grown originally in the presence of cobalamin. The present results, some of which were obtained concurrently with the above work, provide a direct confirmation for a role of cobalamin in methionine synthesis since the free vitamin is active in promoting formation of methionine from homocysteine if the enzymic extracts are made by ultrasonic vibration rather than after drying with acetone. It is probable that treatment with acetone destroys some enzyme system or systems required for the further metabolism of cobalamin itself.

With enzymic extracts of strain PA15 there is no requirement for cobalamin when natural folic acid (provided as an extract of heated organisms) is the source of folic acid; cobalamin is needed, however, when tetrahydro-PtGA or its formaldehyde derivative is substituted for the natural folic acid. These results might suggest a function of cobalamin in the conversion of the simpler reduced forms of folic acid into a coenzyme form. On the other hand, ultrasonic extracts of strain 121/176

(a cobalamin auxotroph) still require cobalamin for the synthesis of methionine in the presence of heated extract of the same organism. The latter extract presumably contains an effective natural form of folic acid since it activates methionine synthesis by enzymic extracts of strain PA 15 in the absence of cobalamin. Extracts of heated suspensions of strain PA 15 did not allow methionine synthesis by the enzyme from strain 121/176 unless cobalamin was added; it is unlikely therefore that the heated extract contained any cobalamin (unpublished experiments of J. R. Guest). It is concluded provisionally that a role of cobalamin (if there is one) in the formation from tetrahydro-PtGA of a folic acid coenzyme cannot be the sole function of the vitamin in the overall methylation of homocysteine, at any rate by *E. coli* 121/176.

Other possibilities for the nature of the function of cobalamin, particularly that it (or a derivative) may act catalytically in the reduction of a compound containing a hydroxymethyl group to one with a methyl group, will not be examined here since they have been fully discussed by Kisliuk & Woods (1960). The present results do not add further to the arguments, but provide a firmer factual background.

The significantly higher activity (compared with cobalamin) of a form of vitamin B_{12} which has coenzyme activity in the metabolism of glutamate by *Clostridium tetanomorphum* (Barker *et al.* 1958; Weissbach *et al.* 1959) adds weight to the view that cobalamin must be transformed into a higher compound before it has functional activity in isolated enzyme systems. A few other analogues of vitamin B_{12} (with a different basic group in the nucleotide residue) had activity equal to or approaching that of cobalamin; it has not yet been determined whether they have intrinsic activity or whether they (and perhaps cobalamin also) are converted into one particular form by enzymes present in the crude extracts used.

Whatever the nature of the source of folic acid coenzyme or of the C_1-unit donor, the effect of increasing cobalamin concentration on methionine synthesis was quantitatively somewhat variable with different enzyme preparations. The overall reaction is undoubtedly complex and such variability may have been due to one or other of the enzymes present in the crude preparations used being in limiting supply in different batches.

Effect of other factors. Tetrahydropteroylglutamic acid almost completely inhibited the synthesis of methionine promoted by ultrasonic extracts of strain PA 15 in the presence of extract of heated organisms (no cobalamin added). This is similar to the effect described and discussed by Kisliuk & Woods (1960), where the activity of natural folic acid coenzyme in extracts of acetone-dried powders not treated with Dowex-1 resin was severely inhibited by tetrahydro-PtGA. No factual explanation has so far been obtained.

A requirement for DPN in the overall reaction showed up clearly in the test systems not containing extract of heated organisms; the latter presumably contains this factor. Both glucose and DPN could be replaced by $DPNH_2$ at concentrations of the same order as those of serine and homocysteine; the main function of glucose is therefore probably in the generation of $DPNH_2$. The reduced form of the coenzyme was not, however, used as a routine since the enzymic extracts had strong $DPNH_2$-oxidase activity. In their enzyme system derived from a cobalamin auxotroph of *E. coli*, Hatch, Takeyama, Cathou, Larrabee & Buchanan (1959) have found that $DPNH_2$ can be replaced (under an atmosphere of hydrogen) by reduced flavin mononucleotide or reduced flavinadenine dinucleotide.

It is clear that glucose, DPN and ATP + Mg^{2+} ions are necessary for the acceptance by homocysteine of the C_1 unit and its reduction to a methyl group; these substances were still required when serine was replaced as donor by tetrahydro-PtGA–CH_2O.

With enzyme systems isolated from animal tissues for the methylation of homocysteine, TPN is more effective than DPN (Nakao & Greenberg, 1958; Stevens & Sakami, 1959). With enzymic extracts of strain PA 15, TPN was inhibitory when the extract of heated *E. coli* was source of folic acid and did not replace DPN (though it was not inhibitory) in the two test systems in which tetrahydro-PtGA or its formaldehyde derivative served (in the presence of cobalamin) as coenzyme. Kisliuk & Woods (1960), using enzyme preparations from acetone-dried organisms, have also found that TPN does not replace DPN and is inhibitory.

SUMMARY

1. Ultrasonic extracts of *Escherichia coli* strain 121/176 (a cobalamin or methionine auxotroph) and of strain PA 15 (a serine or glycine auxotroph) synthesize methionine from homocysteine and serine under certain conditions in the presence of glucose, adenosine triphosphate, magnesium ions, diphosphopyridine nucleotide and a source of natural folic acid (extract of heated organism).

2. With strain 121/176 the further provision of cobalamin is necessary if the organism has been grown originally on a medium devoid of this vitamin. Natural folic acid can be replaced by tetrahydropteroylglutamate.

3. With strain PA 15 cobalamin is not required if the natural source of folic acid coenzyme is

provided. Tetrahydropteroylglutamate can, however, only be used if cobalamin is present.

4. The formaldehyde derivative of tetrahydropteroylglutamate can serve with both strains both as source of folic acid coenzyme and of C_1 units provided again that cobalamin is present.

5. Growth of either strain in the presence of cobalamin enhances the ability of ultrasonic extracts of the organisms to methylate homocysteine.

6. Reduced diphosphopyridine nucleotide abolishes the requirement for both glucose and diphosphopyridine nucleotide.

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Inhibition of Methionine Synthesis in *Escherichia coli* by Analogues of Cobalamin

By J. R. GUEST
Microbiology Unit, Department of Biochemistry, University of Oxford

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A relationship between cobalamin and the synthesis of methionine by *Escherichia coli* was first established by the isolation of auxotrophic strains requiring either cobalamin or methionine for growth (Davis & Mingioli, 1950). Cobalamin has since been found to increase methionine synthesis from homocysteine and serine by washed suspensions of many strains of *E. coli* (Gibson & Woods, 1960); such synthesis is also increased several-fold by the addition of cobalamin to ultrasonic extracts of an auxotrophic strain, *E. coli* 121/176, which requires cobalamin or methionine for growth (Helleiner & Woods, 1956). More recently ultrasonic extracts of another auxotrophic strain (PA 15), which does not require cobalamin for growth, have been found to require the vitamin for methionine synthesis only when the source of the cofactor for the transfer of the C_1 unit is tetrahydropteroylglutamic acid and not when heated extract of the organism is used. With ultrasonic extracts of strain 121/176, cobalamin is essential with both sources of the folic acid cofactor (Helleiner, Kisliuk & Woods, 1957; Guest, Helleiner, Cross & Woods, 1960).

Several analogues of cobalamin have been prepared (Smith, Parker & Gant, 1956) and reported