3. These comparisons suggest that the enzyme, being composed of large molecules with restricted mobility in the substrate, removes a number of adjacent glucose residues from each of relatively few sites of attack.

A portion of this research was conducted under a project authorized by U.S. Public Law no. 480, 83rd Congress.

REFERENCES

- Calvert, M. A. & Clibbens, D. A. (1951). *J. Text. Inst.* **42**, T211.
- Clibbens, D. A., Geake, A. & Ridge, B. P. (1927). *J. Text. Inst.* **18**, T277.
- Cumberbirch, R. J. E. (1955). *Rep. Progr. appl. Chem.* **40**, 451.
- Cumberbirch, R. J. E. & Harland, W. G. (1958). *J. Text. Inst.* **49**, T679.
- Fargher, R. G. (1953). *Symp. biochem. Soc.* **11**, 71.
- Flory, P. J. (1953). *Principles of Polymer Chemistry*, p. 311. New York: Cornell University Press.
- Pigman, W. (1951). In *The Enzymes*, vol. 1, part 2, p. 725. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press Inc.
- Searle, F. (1929). *J. Text. Inst.* **20**, T162.
- Thomas, R. (1956). *Aust. J. biol. Sci.* **9**, 159.
- Walseth, C. S. (1952). *T.A.P.P.I.* **35**, 233.
- Whitaker, D. R. (1953). *Arch. Biochem. Biophys.* **43**, 253.
- Whitaker, D. R. (1957). *Canad. J. Biochem. Physiol.* **35**, 733.
- Whitaker, D. R., Colvin, J. R. & Cook, W. H. (1954). *Arch. Biochem. Biophys.* **49**, 257.

Biochem. J. (1961) **79**, 566

Folic Acid and the Synthesis of Methionine by Extracts of *Escherichia coli*

By K. M. JONES*, J. R. GUEST AND D. D. WOODS
Microbiology Unit, Department of Biochemistry, University of Oxford

(Received 1 December 1960)

The synthesis of methionine from homocysteine and serine by enzymic extracts of *Escherichia coli* has been found in earlier work from this Laboratory to require a cofactor of the folic acid group. In experiments with dialysed and Dowex 1-treated enzymic extracts, the folic acid cofactor can be supplied either by an extract of heated *E. coli*, or under certain conditions by tetrahydropteroylglutamate (Szulmajster & Woods, 1960; Kisliuk & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960). These two sources of cofactor differ in their properties, and in the conditions under which they show activity with extracts of *E. coli* PA15, a glycine or serine auxotroph. First, the cofactor activity of tetrahydropteroylglutamate is rapidly lost on exposure to air, whereas that of the extract of heated *E. coli* is stable under these conditions. Secondly, the utilization of tetrahydropteroylglutamate by extracts of strain PA15 is dependent upon cobalamin. With ultrasonic extracts the addition of cobalamin to the reaction mixtures is sufficient to allow tetrahydropteroylglutamate to act as cofactor (Guest *et al.* 1960), whereas with extracts of acetone-dried organisms it is necessary that the organisms should have been grown in the presence of cobalamin for tetrahydropteroylglut-

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

amate to be effective (Kisliuk & Woods, 1960). On the other hand, when extract of heated *E. coli* is used as the source of cofactor, cobalamin is not needed with either type of enzymic extract. Thirdly, the methionine synthesis promoted by extract of heated *E. coli* is inhibited by tetrahydropteroylglutamate in the absence of cobalamin (Kisliuk & Woods, 1960; Guest *et al.* 1960).

E. coli 121/176 is an auxotroph requiring cobalamin or methionine for growth. Ultrasonic extracts prepared from organisms grown in the presence of methionine differ from those of strain PA15 in that cobalamin is needed for methionine synthesis promoted by either extract of heated *E. coli* or by tetrahydropteroylglutamate.

The observed differences between extract of heated *E. coli* and tetrahydropteroylglutamate in this reaction suggest that the latter is not identical with the factor or factors present in the extract of heated *E. coli*. Although most work with reactions requiring folic acid cofactors has been done with tetrahydropteroylglutamate, experiments with two other such reactions have also shown that this compound is not identical with the active substance or substances in extracts of heated organisms.

Wright (1958) has found seven compounds, all pteridine derivatives, in heated extracts of *Clostridium cylindrosporium* which act as cofactors for the

conversion of serine into glycine by enzyme-containing extracts of *Clostridium sticklandii*. The common requirement for cofactor activity was the presence of three or seven glutamic acid residues, regardless of whether the pteridine part of the molecule was reduced or unreduced, formylated or unformylated. Two of these compounds have been identified as pteroyltriglutamate and N^{10} -formylpteroyltriglutamate. The others are believed to be derivatives of N^6 -formyltetrahydropteroyltriglutamate and N^6 -formyltetrahydropteroylheptaglutamate, each containing additional glycine residues. The remaining compounds are more complex and as yet uncharacterized.

The cofactor activity of heated extracts of *Clostridium acidii-urici* in the decomposition of formimidoylglycine (formiminoglycine) by enzyme-containing extracts of the same organism (Rabinowitz, 1959, and personal communication) is vested entirely in tetrahydropteroyltriglutamic acid. No other active compounds were found.

These findings are in accordance with the general observation that naturally occurring forms of folic acid, whether or not they show cofactor activity for any of these reactions, are very largely triglutamates and heptaglutamates (Wright, 1958; Rabinowitz, 1960). Little, if any, of the monoglutamate forms are found in fresh tissues, though they are formed rapidly from the conjugated compounds during autolysis.

These results suggest that the folic acid components of the extract of heated *E. coli* which are active as cofactors of methionine synthesis may be polyglutamyl compounds. In the present work, the cofactor activities of various synthetic polyglutamates of the folic acid group have been investigated and their properties compared with those of tetrahydropteroyltriglutamate and the extract of heated *E. coli*. A brief report of part of this work has already appeared (Guest & Jones, 1960).

Throughout this paper the term folic acid will be used in general reference to all members of this group of vitamins and growth factors.

MATERIALS AND METHODS

Organisms. Stock cultures of strains of *E. coli* were maintained on slopes of tryptic meat-agar, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°. Most of the work was done with *E. coli* strain PA15, an auxotroph which requires either serine or glycine for growth and was originally obtained from Dr Barbara Wright. *E. coli* strain 121/176, a methionine or cobalamin auxotroph (Davis & Mingioli, 1950), was used in a few experiments; it was obtained from Professor B. D. Davis.

Media and growth. The organisms were grown on solid media in enamelled trays. The media and methods of growth of organisms for the subsequent preparation of ultrasonic and heated extracts have been described by Guest *et al.*

(1960), and for the preparation of acetone-dried powders by Szulmajster & Woods (1960). In those experiments where cobalamin (13 μ m-moles/500 ml. of medium) was present during growth, it was added with the inoculum to the surface of each tray.

Preparation of enzyme-containing extracts. Ultrasonic extracts were prepared by the method of Guest *et al.* (1960). Potassium phosphate buffer (0.05M, pH 7.8) was used throughout for the preparation of suspensions of organisms before ultrasonic treatment and for the dialysis of the extracts.

Extracts of acetone-dried organisms were prepared in general as described by Guest & Woods (1960). The acetone-dried powders, homogenized in water, were, however, subjected to ultrasonic vibration (25 kcyc./sec.; 2.5 min.; 600 w with an ultrasonic generator type E7590B of Mullard Ltd., London, W.C.1) before being centrifuged and dialysed. Compared with extracts made by homogenization only, such treatment increased the total activity extracted per gram of powder about 2.5-fold with only a small decrease in specific activity.

All extracts were treated before use with Dowex 1X8 resin (chloride form; 100–200 mesh) by the method of Kisliuk & Woods (1960). The protein concentration of extracts was estimated spectrophotometrically by the method of Layne (1957).

Extract of heated *Escherichia coli*. This was prepared as described by Guest *et al.* (1960) and contained 14 mg. dry wt. of solid material/ml.

Test for methionine synthesis. The basal reaction mixture used in experiments with ultrasonic extracts contained (μ moles in a final volume of 2 ml.): potassium phosphate buffer, pH 7.8, 250; DL-homocysteine, 10; L-serine, 10; hexose diphosphate, 15 or glucose, 20; $MgCl_2$, 10; adenosine triphosphate (ATP), 10; diphosphopyridine nucleotide (DPN), 1; pyridoxal phosphate, 0.5. In experiments with extracts of acetone-dried organisms DL-homocysteine was increased to 20 μ moles and pyridoxal phosphate to 1 μ mole. The amount of enzyme-containing extract used contained 10 mg. of protein.

Reaction mixtures were incubated at 37° for 2–4 hr. in an atmosphere of H_2 . After heating at 100° for 3 min. the reaction mixtures were centrifuged and the supernatant fluids used for the estimation of methionine.

Methionine was assayed microbiologically with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 by the method described by Gibson & Woods (1960). DL-Methionine was used as standard; the organism responds only to the L-isomer.

Estimation of folic acid. Microbiological assays with *Lactobacillus casei* (A.T.C.C. no. 7469), *Streptococcus faecalis* (A.T.C.C. no. 8043) and *Pediococcus cerevisiae* (*Leuconostoc citrovorum*) (A.T.C.C. no. 8081) were employed for the estimation of various forms of folic acid. *Lactobacillus casei* grows with mono-, di- and tri-glutamate forms of folic acid but not with pteroate; *S. faecalis* responds to pteroate, pteroyltriglutamate and their derivatives but not to di- and tri-glutamate; *P. cerevisiae*, however, requires a reduced form of folic acid, showing no response with any unreduced compound. Under the conditions of the assay only reduced compounds substituted in the N^6 -position would be expected to be stable. The response of *P. cerevisiae* to reduced triglutamate compounds is uncertain (Rabinowitz, 1960). None of the organisms responds to pteroylheptaglutamate.

The three organisms were maintained on medium B1 of Nimmo-Smith, Lascelles & Woods (1948). Stab cultures were transferred fortnightly, incubated for 16 hr. at 37° and stored at 4°.

A single basal medium, based upon that of Toennies, Frank & Gallant (1952), was used for all three organisms. It contained (in each litre of final medium): tryptic digest of casein, 500 ml.; DL-alanine, 200 mg.; L-asparagine, 100 mg.; L-cysteine, 200 mg.; glucose, 20 g.; KH_2PO_4 , 0.1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg.; NaCl, 10 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg.; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg.; adenine, 10 mg.; guanine, 10 mg.; xanthine, 10 mg.; uracil, 10 mg.; nicotinic acid, 0.5 mg.; calcium pantothenate, 0.5 mg.; thiamine hydrochloride, 0.5 mg.; riboflavin, 0.5 mg.; pyridoxamine hydrochloride, 0.2 mg.; *p*-aminobenzoic acid, 0.1 mg.; biotin, 10 μg .; pH adjusted to 6.4–6.5. The tryptic digest of casein was prepared as described by Roberts & Snell (1946) and modified by Nimmo-Smith *et al.* (1948). The medium was normally made up with the quantities above in a volume of 700 ml., and then diluted to the correct strength during the preparation of the assays.

The strong basal medium (3.5 ml.), test samples and water to a total volume of 4.9 ml. were distributed in 125 mm. $\times$ 16 mm. tubes. Standards containing pteroylglutamic acid, pteroyltriglutamic acid or leucovorin (synthetic *N*⁵-formyltetrahydropteroyltriglutamic acid) (0.05–0.5 μM) were included in each assay. Leucovorin is a mixture of two isomers, only one of which supports growth of the assay organisms. The tubes were autoclaved for 2.5 min. at 121°. The inoculum was derived from a culture grown in medium B2 of Nimmo-Smith *et al.* (1948) for 16 hr. at 37°, harvested and washed with water. Inoculum development tubes, containing 5 ml. of basal medium supplemented with pteroyltriglutamic acid or leucovorin (0.3 μM), were inoculated with an amount of washed organisms equivalent to about 0.1 mg. dry wt. and incubated for 4–6 hr. at 37°, by which time the cultures were in the logarithmic phase of growth. This culture was centrifuged and the organisms were suspended in the culture volume of water. A tenfold dilution of this suspension was used as the inoculum for the assays (0.1 ml./tube).

Tubes were incubated upright in air for 20–22 hr. at 37°. Growth was assessed in a Hilger Spekker Photoelectric Absorptiometer fitted with a neutral filter (H 508). The uninoculated medium was used as a blank.

The method used to degrade polyglutamates of the folic acid group to the corresponding monoglutamates was that recommended by the Association of Vitamin Chemists Inc. (1951) with a conjugase preparation from hog kidney. The final filtrate, containing about 4 μM -moles of folic acid/ml., was adjusted to pH 7, suitably diluted, and used for assay with the various organisms. The efficacy of the conjugase treatment was checked by a control containing a known amount of pteroyltriglutamate. The results were corrected for the folic acid content of the hog-kidney extract (about 25 μM -moles/ml.).

Chemicals. Pteroyl- γ -glutamyl- γ -glutamylglutamic acid (generally referred to as pteroyltriglutamate) was a gift from Dr E. L. R. Stokstad. For some experiments it was purified by chromatography on a column of cellulose powder with 0.1M-sodium phosphate buffer, pH 7.0, saturated with isoamyl alcohol at 25°, as solvent. The purified material was precipitated several times from neutral solution with acid and finally freeze-dried.

*N*¹⁰-Formylpteroyltriglutamate was prepared by dissolving pteroyltriglutamate (100 mg.) in 4 ml. of 98% formic acid and keeping it in the dark for 48 hr. The formic acid was then removed by freeze-drying, and the product dissolved in water and again freeze-dried. This gave a yellow material, which was purified by repeated solution in water and precipitation with acid.

A solution of pteroylheptaglutamic acid was obtained as a gift from Dr E. Kodicek. Its purity was checked by microbiological assay. The amount of active material was only about half that originally present in the solution but 95% of this was inactive for both *S. faecalis* R and *Lactobacillus casei* before conjugase treatment and active for both organisms after treatment with conjugase.

Tetrahydropteroyltriglutamic acid was prepared from 0.5 or 1.0 g. of pteroyltriglutamic acid by the method of Kisliuk & Woods (1960) and stored as the solid under H_2 .

Tetrahydropteroyltriglutamic acid and *N*¹⁰-formyltetrahydropteroyltriglutamic acid were prepared as solutions immediately before use by the following method. Platinic oxide catalyst (Adam's catalyst) (2–5 mg.) was suspended in 0.02M-sodium phosphate buffer, pH 7.0, in the main vessel of a Warburg manometer. Pteroyltriglutamic acid or *N*¹⁰-formylpteroyltriglutamic acid (4–16 μM -moles), dissolved in 0.02M-sodium phosphate buffer and adjusted to pH 7.0, was placed in the side arm. The manometer vessel, protected from light with a bag of black material, was filled with H_2 and shaken in a water bath at 37°. The uptake of H_2 by the catalyst was allowed to proceed to completion (usually 30–40 min.). The folic acid compound was then added and the mixture again shaken at 37° until an amount of H_2 corresponding to 2 moles of H_2 /mole of the folic acid compound had been taken up (30–90 min.). The solution of reduced pteridine (1–5 mm) was withdrawn by a Pasteur pipette with a plug of cotton wool in its tip to filter off the suspended catalyst. The solution was then immediately added to the reaction mixtures for testing methionine synthesis.

*N*⁵-Formyltetrahydropteroyltriglutamate was prepared by a modification of the method used for the synthesis of the corresponding monoglutamate by Shive, Bardos, Bond & Rogers (1950) and Ericson (1953). A solution of *N*¹⁰-formyltetrahydropteroyltriglutamate was prepared as described above and 2-mercaptoethanol (final concentration 0.4M) added. The solution was adjusted to pH 8.2 and autoclaved for 1 hr. at 121°. After cooling and neutralizing, the solution contained *N*⁵-formyltetrahydropteroyltriglutamate in 20–40% yield. The compound was purified either by chromatography on triethylaminoethylcellulose with a gradient from water to 0.5M-potassium phosphate buffer, pH 6.1, or by chromatographing on Whatman no. 3 paper, with, as solvent, 0.24M-citric acid brought to pH 9.0 with ammonia and saturated with isoamyl alcohol at 25°. The amount of *N*⁵-formyltetrahydropteroyltriglutamic acid in the product was estimated (a) by microbiological assay with *P. cerevisiae* before and after conjugase treatment and (b) by the increase in absorption at 350 m μ when the solution was adjusted to pH 1.0 (Rabinowitz, 1960).

Tetrahydropteroylheptaglutamic acid was prepared by adjusting the solution of pteroylheptaglutamic acid (containing 5 μM -moles) to 0.02M and pH 7.0 with sodium phosphate buffer, adding 2 mg. of platinic oxide catalyst, and shaking for 1 hr. at room temperature in an atmosphere of H_2 . The amount of material available was too small to allow the H_2 uptake to be measured manometrically.

Cobalamin was crystalline material obtained from British Drug Houses Ltd., Poole, Dorset; it was about 90% pure by spectrophotometric assay. ATP and DPN were products of 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

Methionine synthesis by ultrasonic extracts of *Escherichia coli* PA 15

Extract of heated Escherichia coli. With dialysed and Dowex 1-treated ultrasonic extracts of *E. coli* PA 15, extract of heated organisms is able to act as the source of folic acid cofactor for methionine synthesis and its activity is unaffected by added cobalamin (Guest *et al.* 1960).

The folic acid content of a freshly prepared batch of extract of heated organisms has been measured by microbiological assay with the three test organisms both before and after treatment with a conjugase preparation (Table 1). Much the greatest part of the folic acid was in the form of polyglutamates, since a very large increase in activity for both *S. faecalis* and *P. cerevisiae* was observed on conjugase treatment. The increase in activity for *Lactobacillus casei* on conjugase treatment suggests that a portion of the polyglutamates contain more than three glutamic acid residues.

With a sample of extract of heated organisms of known folic acid content, the amount of this extract required for half-maximum methionine synthesis was determined. The folic acid concentration of a reaction mixture containing this amount of extract of heated organisms is given in Table 1. The values were determined with the three assay organisms after conjugase treatment of the heated extract.

The cofactor activity of extract of heated organisms is inhibited by tetrahydropteroylglutamate (Kisliuk & Woods, 1960; Guest *et al.* 1960). This

inhibition has now been examined quantitatively by the method of Lineweaver & Burk (1934) and was found to be competitive (Fig. 1). The K_i for etrtahydropteroylglutamate was 2.2 μ M.

Tetrahydropteroylglutamate. This compound is only active as a cofactor of methionine synthesis by ultrasonic extracts in the presence of added cobalamin (Guest *et al.* 1960). The K_m for tetrahydropteroylglutamate in the presence of 70 μ moles of cobalamin was 180 μ M.

Tetrahydropteroyltriglutamate. In contrast with the monoglutamate, this substance showed cofactor activity in the absence of added cobalamin and its activity was not appreciably affected by the addition of cobalamin (70 μ moles) to the reaction mixtures (Fig. 2). The K_m for tetrahydropteroyl-

Fig. 1. Effect of tetrahydropteroylglutamate on methionine synthesis by ultrasonic extracts of *E. coli* PA15 with extract of heated organisms as cofactor. Incubation time, 2 hr. *S*, amount of extract of heated organisms (ml.); *V*, methionine formation (μ m-moles of L-methionine/mg. of protein). Concentration of tetrahydropteroylglutamate: O, nil; $\bullet$, 10 μ M; $\bullet$, 20 μ M.

Table 1. Folic acid content of extract of heated *Escherichia coli*

A freshly prepared sample of extract of heated *E. coli* was used. Conjugase treatment, microbiological assays and tests of the synthesis of methionine were performed as described in the Methods section. The concentration of heated extract in the reaction mixture required for half-maximal methionine synthesis was determined by the method of Lineweaver & Burk (1934). The concentrations of folic acid represent the values obtained with the different organisms after conjugase treatment of the heated extract. The three test organisms react to different forms of folic acid (see Methods section).

Assay organism	Concn. of folic acid (μ M)		
	In heated extract		Required for half-maximum methionine synthesis
	Before conjugase treatment	After conjugase treatment	
<i>Lactobacillus casei</i>	29	44	1.5
<i>Streptococcus faecalis</i> R	1.0	90	3.0
<i>Pediococcus cerevisiae</i>	1.1	12	0.4

triglutamate was $8 \mu\text{M}$, and was not affected by the presence of cobalamin.

The activity of tetrahydropteroyltriglutamate, like that of extract of heated organisms, was inhibited competitively by tetrahydropteroylglutamate (Fig. 3). The K_i for tetrahydropteroylglutamate was $5.3 \mu\text{M}$. Tetrahydropteroyltriglutamate did not inhibit the cofactor activity of the extract of heated *E. coli*.

Similar results were obtained with the tetrahydro derivative prepared either from purified synthetic pteroyltriglutamate or from pteroyltriglutamate isolated from natural sources.

Fig. 2. Tetrahydropteroyltriglutamate as a cofactor of methionine synthesis by ultrasonic extracts of *E. coli* PA15. Incubation time, 2 hr. ■, Without cobalamin; □, with cobalamin ($70 \mu\mu\text{moles}$).

Fig. 3. Effect of tetrahydropteroylglutamate on methionine synthesis by ultrasonic extracts of *E. coli* PA15 with tetrahydropteroyltriglutamate as cofactor. Incubation time, 2 hr. S , Tetrahydropteroyltriglutamate concentration (μM); V , L-methionine formed ($\mu\text{m-moles/mg. of protein}$). Concentration of tetrahydropteroylglutamate: ○, nil; ●, $15 \mu\mu\text{M}$; ◐, $30 \mu\mu\text{M}$.

N¹⁰-Formyltetrahydropteroyltriglutamate. This folic acid derivative was also active in promoting methionine synthesis in the absence of cobalamin and was unaffected by added cobalamin. The activity of the *N¹⁰*-formyl derivative was approximately the same as that of tetrahydropteroyltriglutamate; the K_m was $8 \mu\text{M}$. Its cofactor activity was also inhibited by tetrahydropteroylglutamate, but the nature of the inhibition has not been examined. *N¹⁰*-Formyltetrahydropteroyltriglutamate does not itself inhibit methionine synthesis with extract of heated organisms as cofactor.

When serine was omitted from the reaction mixture and *N¹⁰*-formyltetrahydropteroyltriglutamate increased to $2.5 \mu\text{moles}$, no methionine was formed. This formyl group was evidently incapable of acting as a source of C_1 units for methionine formation.

N⁵-Formyltetrahydropteroyltriglutamate. This compound also showed cofactor activity which was entirely independent of added cobalamin; the K_m was $1.6 \mu\text{M}$ (Fig. 4).

Tetrahydropteroylglutamate acted as a competitive inhibitor of *N⁵*-formyltetrahydropteroyltriglutamate in methionine synthesis; the K_i was $1.9 \mu\text{M}$. Like the other triglutamate derivatives, *N⁵*-formyltetrahydropteroyltriglutamate did not inhibit the activity of the heated extract of *E. coli*.

The formyl group of this compound, like that of the *N¹⁰*-formyl derivative, was unable to serve as the source of the C_1 unit for methionine synthesis in the absence of serine.

Fig. 4. *N⁵*-Formyltetrahydropteroyltriglutamate as cofactor of methionine synthesis by ultrasonic extracts of *E. coli* PA15. Incubation time, 2 hr. ●, Without cobalamin; ○, with cobalamin ($70 \mu\mu\text{moles}$).

Tetrahydropteroylheptaglutamate. The reduced solution of pteroylheptaglutamate, presumed to contain tetrahydropteroylheptaglutamate, showed cofactor activity for methionine synthesis at concentrations of the same order of magnitude as were active with tetrahydropteroyltriglutamate.

Unreduced folates. Pteroyltriglutamate, pteroyltriglutamate, and N^{10} -formylpteroyltriglutamate showed no cofactor activity in the synthesis of methionine, nor did they affect the activity of the heated extract of organisms.

Effect of cobalamin on the inhibition caused by tetrahydropteroyltriglutamate. The inhibition of methionine formation caused by tetrahydropteroyltriglutamate when polyglutamate compounds served as the folic acid cofactors was overcome by cobalamin (Table 2). Tetrahydropteroyltriglutamate, however, is itself an active cofactor under these conditions and the nature of the action of cobalamin is still unknown.

Stability of the active compounds. The cofactor activity of the extract of heated organisms is stable for several months in air at -14° . Incubation for 18 hr. at 30° in air caused a 10–15% loss of activity, whereas the activity of tetrahydropteroyltriglutamate was completely destroyed under these con-

ditions (Table 3). Of the reduced triglutamates the N^8 - and N^{10} -formyltetrahydropteroyltriglutamates were as stable as the heated extract, but the activity of tetrahydropteroyltriglutamate was considerably reduced and resembled the monoglutamate in this respect (Table 3).

Methionine synthesis by extracts of acetone-dried Escherichia coli PA 15

The cofactor activity of some of the above compounds has also been examined qualitatively, though not quantitatively, by using extracts of acetone-dried organisms of *E. coli* PA 15 as source of the enzymes for methionine synthesis. With such extracts tetrahydropteroyltriglutamate is only active when the organisms have been grown originally in the presence of cobalamin; extract of heated *E. coli* on the other hand supports methionine synthesis whether or not the organisms used in making the acetone-dried powder have been grown with cobalamin. Tetrahydropteroyltriglutamate and its N^8 -formyl derivatives were active with extracts of acetone-dried organisms grown in the absence of cobalamin; that is, they resemble the heated extract of *E. coli* rather than tetrahydropteroyltriglutamate (Table 4).

Table 2. *Effect of cobalamin on the inhibition of the cofactor activity of several conjugated forms of folic acid by tetrahydropteroyltriglutamate*

Reaction mixtures contained 1 μ mole of tetrahydropteroyltriglutamate, 0.7 μ m-mole of cobalamin and 0.1 ml. of the extract of heated *E. coli* when added. Incubation was for 2 hr.

Source of folic acid cofactor	L-Methionine formed (μ m-moles/mg. of protein)		
	No additions	Tetrahydropteroyltriglutamate	Tetrahydropteroyltriglutamate + cobalamin
None	2	2	58
Extract of heated <i>E. coli</i>	68	4	63
Tetrahydropteroyltriglutamate (0.5 mM)	96	4	90
N^{10} -Formyltetrahydropteroyltriglutamate (0.5 mM)	96	3	93
N^8 -Formyltetrahydropteroyltriglutamate (0.1 mM)	99	3	94

Table 3. *Stability of folic acid cofactors*

Samples of the folic acid compounds in 0.02M-phosphate buffer, pH 7.0, and of extracts of heated *E. coli* were incubated at 30° in air for 18 hr. at the stated concentrations. The reaction mixture for testing tetrahydropteroyltriglutamate both before and after treatment contained 0.7 μ m-mole of cobalamin. Incubations were for 2 hr. with ultrasonic extracts of *E. coli* PA15.

Source of folic acid cofactor	Concn. of folic acid cofactor		L-Methionine formed (μ m-moles/mg. of protein)	
	During treatment in air (mM)	During test for methionine formation (μ M)		
			Untreated	Treated
Tetrahydropteroyltriglutamate	5.0	300	40	0
Tetrahydropteroyltriglutamate	0.2	15	51	12
N^{10} -Formyltetrahydropteroyltriglutamate	0.2	15	50	43
N^8 -Formyltetrahydropteroyltriglutamate	0.5	2.4	60	53
Extract of heated <i>E. coli</i>	0.01–0.09*	0.45–3.4*	58	51

* Estimated microbiologically with three organisms after treatment with conjugase.

*Methionine synthesis by ultrasonic extracts of
E. coli 121/176*

When ultrasonic extracts of methionine-grown *E. coli* 121/176, a cobalamin or methionine auxotroph, were employed, the extract of heated organisms, tetrahydropteroylglutamate and tetrahydropteroyltriglutamate all required the addition of cobalamin to the reaction mixtures before methionine synthesis exceeded about 20% of the maximum (Table 5). Inhibition of the activity of the heated extract and the derivatives of tetrahydropteroyltriglutamate by tetrahydropteroylglutamate was not found with ultrasonic extracts of this strain; cobalamin must be present for optimum synthesis of methionine and cobalamin is known to prevent such inhibition with ultrasonic extracts of strain PA15. However, the inhibitory properties of tetrahydropteroylglutamate may be inferred from the fact that in the absence of cobalamin the value obtained for methionine formation in strain 121/176 is least with tetrahydropteroylglutamate as the cofactor. In addition

the value with extract of heated organisms as the cofactor was decreased by addition of the reduced monoglutamate.

DISCUSSION

Qualitative comparison of the results obtained with the synthetic compounds, tetrahydropteroyltriglutamate, *N*¹⁰-formyltetrahydropteroyltriglutamate and *N*⁵-formyltetrahydropteroyltriglutamate, with those obtained with heated extract of *E. coli* and with tetrahydropteroylglutamate shows that the three triglutamates resemble in behaviour the heated extract rather than tetrahydropteroylglutamate. First, they are quite independent of cobalamin for their cofactor activity in methionine synthesis, except in extracts of strain 121/176 where cobalamin is also required for the heated extract to be active. Secondly, their activity is inhibited competitively by tetrahydropteroylglutamate.

A quantitative comparison of the cofactor activities of the synthetic compounds (Table 6) confirms the similarity of the triglutamates to the

Table 4. *Folic acid cofactors in methionine synthesis by extracts of acetone-dried Escherichia coli PA15*

Incubations were performed as described in the Methods section for 3 hr.

Folic acid compound	L-Methionine formed (μ m-moles/mg. of protein) by extracts prepared from acetone-dried organisms grown	
	Without cobalamin	With cobalamin
Tetrahydropteroylglutamate (0.5 mM)	3	25
Tetrahydropteroyltriglutamate (75 μ M)	45	33
<i>N</i> ⁵ -Formyltetrahydropteroyltriglutamate (5 μ M)	40	—
Extract of heated <i>E. coli</i> (0.1 ml.)	36	35

Table 5. *Effect of cobalamin on methionine synthesis by ultrasonic extracts of Escherichia coli 121/176 with various folic acid cofactors*

Incubation was for 4 hr. Reaction mixtures contained 70 μ moles of cobalamin where indicated.

Folic acid compound	L-Methionine formed (μ m-moles/mg. of protein)	
	Without cobalamin	With cobalamin
Tetrahydropteroylglutamate (500 μ M)	0	91
Tetrahydropteroyltriglutamate (150 μ M)	23	128
Extract of heated <i>E. coli</i> (0.15 ml.)	17	80

Table 6. *Concentrations of folic acid cofactors required for half-maximal methionine synthesis by ultrasonic extracts of Escherichia coli PA15*

Values obtained from results given in Table 1, Figs. 2 and 4, and in the text.

Cofactor	Concn. for half-maximal methionine synthesis (μ M)
Tetrahydropteroylglutamate	180
Tetrahydropteroyltriglutamate	8
<i>N</i> ¹⁰ -Formyltetrahydropteroyltriglutamate	8
<i>N</i> ⁵ -Formyltetrahydropteroyltriglutamate	1.6
Extract of heated <i>E. coli</i>	0.4-3.0

active components of the extract of heated *E. coli*. During the reduction of the pteridine ring two stereoisomers are formed by the introduction of a new asymmetric centre at C-6. It is possible that only one of these is active as a cofactor and in this event the concentrations given for the synthetic compounds in Table 6 should be halved. Although the total folic acid concentration corresponding to the amount of heated extract of *E. coli* required for half-maximum methionine synthesis depends to some extent upon the assay organism used, it is clearly of the order of $1\ \mu\text{M}$. There is also the possibility that some of this folic acid may be in forms devoid of cofactor activity and that the true figure for half-maximal activity should be lower. Of the synthetic compounds tested, N^5 -formyltetrahydropteroyltriglutamate was active at concentrations similar to those found in the heated extract. The stability of this compound also resembled that of the heated extract and it may therefore be a component of the extract of heated *E. coli*. Tetrahydropteroyltriglutamate on the other hand was considerably less stable than the heated extract. The N^{10} -formyl derivative was only one-fifth as active as the N^5 -formylated compound, but it appeared to be as stable as the latter. The N^{10} -formyl derivative of tetrahydropteroylmonoglutamate is particularly unstable (Rabinowitz, 1960); the apparent stability of the triglutamate derivative may be due to the conversion of a small portion into the more active N^5 -formyltetrahydropteroyltriglutamate.

In view of the results of Wright (1958) who found that the unreduced pteridine compounds, pteroyltriglutamate and N^{10} -formylpteroyltriglutamate were active in supporting the serine-glycine interconversion in *C. sticklandii*, it is noteworthy that only the reduced compounds are active for methionine synthesis. It seems probable that extracts of *C. sticklandii* were capable of bringing about the reduction enzymically under the conditions used.

The fact that not only tetrahydropteroyltriglutamate but also both its N^5 - and N^{10} -formyl derivatives are active as cofactors raises the question of their mechanism of action. For tetrahydropteroyltriglutamate the suggestion has been made (Kisliuk & Woods, 1960) that $N^{5,10}$ -methylenetetrahydropteroyltriglutamate, which is known to be formed from serine and tetrahydropteroylglutamate (Blakley, 1958), transfers the methylene group to homocysteine, with an accompanying reduction, to form methionine. A similar mechanism might apply to tetrahydropteroyltriglutamate, but the formylated derivatives would have first to be deformylated before this mechanism could operate. If this were the case it is difficult to see why N^5 -formyltetrahydropteroyltriglutamate should be active at con-

centrations one-fifth of those required for tetrahydropteroyltriglutamate. For the N^{10} -formyl compound, which is active at the same concentration as the unsubstituted triglutamate, deformylation or conversion into the N^5 -formyl compound may be a necessary prerequisite for cofactor activity.

It appears possible that the N^5 -formyl group is concerned with the attachment of the folic acid cofactor to the apoenzyme and that it is the N^{10} -position which is active in the transfer of the C_1 unit. It may be suggested that N^5 -formyltetrahydropteroyltriglutamate accepts a C_1 unit from serine at the hydroxymethyl level of oxidation forming N^5 -formyl- N^{10} -hydroxymethyltetrahydropteroyltriglutamate and that this latter compound transfers the hydroxymethyl group to homocysteine with an accompanying reduction, to yield methionine.

It is not surprising that neither of the formyl derivatives of tetrahydropteroyltriglutamate is able to act as donor of a C_1 unit in methionine synthesis when it is remembered that in these compounds the C_1 unit is at a formyl, that is formic acid, level of oxidation whereas the C_1 transfer from serine to yield methionine occurs at the hydroxymethyl (formaldehyde) level.

Cobalamin is required for methionine formation by extracts of *E. coli* 121/176 with the reduced triglutamates or the extract of heated organisms as well as with tetrahydropteroylglutamate as the source of cofactor for C_1 transfer. This supports the view that cobalamin is concerned in the reduction on transfer of C_1 units and not in the elaboration of a competent folic acid coenzyme from tetrahydropteroyltriglutamate.

SUMMARY

1. Tetrahydropteroyltriglutamate, its N^5 - and N^{10} -formyl derivatives and an extract of heated *Escherichia coli* are each able to serve as source of folic acid cofactor for methionine synthesis by ultrasonic extracts of *E. coli* PA 15 in the absence of added cobalamin. Tetrahydropteroylglutamate requires added cobalamin for activity.

2. Pteroylglutamate, pteroyltriglutamate, and N^{10} -formylpteroyltriglutamate are devoid of cofactor activity.

3. The K_m values for the synthetic compounds are: tetrahydropteroylglutamate (with $77\ \mu\text{M}$ moles of cobalamin), $180\ \mu\text{M}$; tetrahydropteroyltriglutamate and its N^{10} -formyl derivative, both $8\ \mu\text{M}$; N^5 -formyltetrahydropteroyltriglutamate, $1.6\ \mu\text{M}$. The folic acid concentration of a reaction mixture giving half-maximal methionine synthesis with extract of heated *E. coli* is $0.4\text{--}3\ \mu\text{M}$, depending upon the assay organism employed.

4. Tetrahydropteroylglutamate is a competitive inhibitor of the cofactor activity of extract of heated *E. coli*, and of tetrahydropteroyltriglutamate and its *N*⁵-formyl derivative. It also inhibits the activity of the *N*¹⁰-formyl compound. In all cases the inhibition is overcome by the addition of cobalamin.

5. The cofactor activity of tetrahydropteroylglutamate and tetrahydropteroyltriglutamate is unstable to exposure to air at 30° for 18 hr., whereas the activities of extract of heated *E. coli* and of *N*¹⁰- and *N*⁵-formyltetrahydropteroyltriglutamate are relatively stable under these conditions.

6. Cobalamin is required for optimum formation of methionine by ultrasonic extracts of *E. coli* 121/176 (a cobalamin- or methionine-requiring auxotroph) when the derivatives of pteroyltriglutamate, tetrahydropteroylglutamate or an extract of heated organisms supply the necessary folic acid cofactor.

One of us (J. R. G.) is indebted to the Medical Research Council for a Scholarship. We are grateful to the Nuffield Foundation for a grant towards the expenses of this work. The work was also aided by grants to the Department from the Rockefeller Foundation and from the United States Department of Health, Education and Welfare.

REFERENCES

- Association of Vitamin Chemists Inc. (1951). *Methods of Vitamin Assay*, 2nd ed., p. 231. New York: Interscience Publishers Inc.
- Blakley, R. L. (1958). *Nature, Lond.*, **182**, 1719.
- Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.
- Ericson, L.-E. (1953). *Ark. Kemi*, **6**, 503.
- Gibson, F. & Woods, D. D. (1960). *Biochem. J.* **74**, 160.
- Guest, J. R., Helleiner, C. W., Cross, M. J. & Woods, D. D. (1960). *Biochem. J.* **76**, 396.
- Guest, J. R. & Jones, K. M. (1960). *Biochem. J.* **75**, 12P.
- Guest, J. R. & Woods, D. D. (1960). *Biochem. J.* **77**, 422.
- Kisliuk, R. L. & Woods, D. D. (1960). *Biochem. J.* **75**, 467.
- Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 451. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Nimmo-Smith, R. H., Lascelles, J. & Woods, D. D. (1948). *Brit. J. exp. Path.* **29**, 264.
- Rabinowitz, J. C. (1959). *Fed. Proc.* **18**, 306.
- Rabinowitz, J. C. (1960). In *The Enzymes*, vol. 2, p. 185. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
- Roberts, E. C. & Snell, E. E. (1946). *J. biol. Chem.* **163**, 499.
- Shive, W., Bardos, T. J., Bond, T. J. & Rogers, L. L. (1950). *J. Amer. chem. Soc.* **72**, 2817.
- Szulmajster, J. & Woods, D. D. (1960). *Biochem. J.* **75**, 3.
- Toennies, G., Frank, H. G. & Gallant, D. L. (1952). *Growth*, **16**, 287.
- Wright, B. E. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **11**, 266.

Biochem. J. (1961) **79**, 574

On the Mechanism of Activation of Phosphoglucomutase by Metal Ions

By C. MILSTEIN*

Department of Biochemistry, University of Cambridge

(Received 1 November 1960)

Phosphoglucomutase is a widely distributed enzyme which catalyses the interconversion of glucose 1-phosphate and glucose 6-phosphate; it requires a coenzyme, glucose 1:6-diphosphate (Cardini, Paladini, Caputto, Leloir & Trucco, 1949). The enzyme has been purified from rabbit skeletal muscle (Najjar, 1948; Jagannathan & Luck, 1949).

Cori & Cori (1937) first showed that Mg²⁺ ion activates the enzymic conversion of glucose 1-phosphate into glucose 6-phosphate. With the extracted enzyme, both as crude extracts and purified preparations, different authors reported differing

effects of Mg²⁺ ion. According to Stickland (1949) and Jagannathan & Luck (1949) no significant activity is present in the absence of Mg²⁺ ion, Mn²⁺ ion or other bivalent metals. Cori, Colowick & Cori (1938) and Najjar (1948), on the other hand, reported that the enzyme is partially active in the absence of added metal, but that activation is obtained by adding Mg²⁺ ion in optimum concentrations (15-fold activation with electrodialysed crude extracts; fourfold activation with the purified enzyme).

The effect of several other metals has also been reported. In crude muscle extracts, Stickland (1949) claimed a further activating effect of a

* Present address: Instituto Nacional de Microbiología, Av. Vélez Sarsfield 563, Buenos Aires, Argentina.