Analogues of Folic Acid and the Activity of Dihydrofolic Acids in the Synthesis of Methionine by *Escherichia coli*

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The term folic acid or folate will be used in general reference to both simple and conjugated members of this family of compounds. Specific compounds will be referred to by names derived from those in common use, e.g. tetrahydropteroylmonoglutamate and dihydropteroyltriglutamate.

The synthesis of methionine from homocysteine and serine or formaldehyde by soluble extracts of *Escherichia coli* PA 15 has been found to require a cofactor of the folic acid group. This can be supplied by tetrahydropteroylmonoglutamate, tetrahydropteroyltriglutamate and its N^5 - and N^{10} -formyl derivatives, or by an extract of heated *E. coli* (Szulmajster & Woods, 1960; Kisliuk & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960; Jones, Guest & Woods, 1961). In order that tetrahydropteroylmonoglutamate may serve as the cofactor for transferring the C_1 unit it is necessary to add cobalamin either to the reaction mixture or to the medium used for the growth of the organisms (Kisliuk & Woods, 1960; Guest *et al.* 1960; Guest & Woods, 1960). Furthermore, in the absence of cobalamin, tetrahydropteroylmonoglutamate inhibits competitively the function both of tetrahydropteroyltriglutamate and its derivatives and of the cofactors present in the extract of heated *E. coli* (Jones *et al.* 1961). However, tetrahydropteroylmonoglutamate and its N^5 -formyl derivative appear to act directly as cofactors when soluble extracts of mammalian liver are used as the source of enzymes for the synthesis of methionine (Nakao & Greenberg, 1958; Stevens & Sakami, 1959).

The detailed mechanism of reduction of a hydroxymethyl group to a methyl group in methionine formation is not known, though mediation by reduced pyridine nucleotide or by reduced flavin derivatives has been proposed (Kisliuk & Woods, 1960; Hatch, Takeyama, Cathou, Larrabee & Buchanan, 1959). In a comparable reaction, the synthesis of thymidylate from deoxyuridylate and formaldehyde by extracts of rat thymus gland, it has been suggested that tetrahydropteroylmonoglutamate serves not only as the carrier of the C_1 unit but also as the reducing agent for methyl-group formation (Humphreys & Greenberg, 1958).

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Evidence was obtained for the production of one methyl group by the oxidation of one molecule of tetrahydropteroylmonoglutamate to dihydropteroylmonoglutamate, which could be reduced back to tetrahydropteroylmonoglutamate by reduced diphosphopyridine nucleotide. Support for this mechanism was obtained by Friedkin (1959), using tritiated tetrahydropteroylmonoglutamate and an enzyme from *E. coli*; the methyl group of thymidylate was labelled with tritium. A similar mechanism is also probable for the synthesis of the methyl groups of choline (Venkataraman & Greenberg, 1958; Peters & Greenberg, 1958). Such a reaction is dependent either upon the presence of substrate concentrations of tetrahydropteroylmonoglutamate or upon its regeneration, presumably by reduced pyridine nucleotide in the presence of dihydrofolate reductase. The latter enzyme is inhibited non-competitively by the folic acid analogues aminopterin and amethopterin (Osborn, Freeman & Huennekens, 1958; Futterman, 1957). In the presence of catalytic concentrations of tetrahydropteroylmonoglutamate these substances would therefore be expected to inhibit methyl-group formation.

The present work was undertaken to test the activity of dihydrofolic acid derivatives as cofactors in the synthesis of methionine by extracts of *E. coli* PA 15; the effect of aminopterin and amethopterin was investigated in order to determine whether the mechanism of methyl-group formation was similar to that proposed for the synthesis of the methyl groups of thymidylate and choline. Since this work was completed convincing evidence (including inhibition by aminopterin) has been obtained that tetrahydropteroylmonoglutamate is the immediate reducing agent in the formation of the methyl group of thymidylate by extracts of *Streptococcus faecalis* (McDougall & Blakley, 1961).

Very recently also Wilmanns, Rücker & Jaenicke (1960) have shown that tetrahydropteroylmonoglutamate is the reducing agent for the formation of the methyl group of methionine by extracts of pig liver. In addition, N^5 -methyl-dihydropteroylmonoglutamate has been tentatively proposed as the structure of an intermediate in this reaction.

METHODS AND MATERIALS

Abbreviations. The following abbreviations, based on PtG for pteroylglutamate, will be used in the remainder of the paper: pteroyltriglutamate (PtG₃), tetrahydropteroylmonoglutamate (H₄PtG), dihydropteroylmonoglutamate (H₂PtG), tetrahydropteroyltriglutamate (H₄PtG₃), dihydropteroyltriglutamate (H₂PtG₃). Derivatives of these compounds will be referred to as, for example, N⁵-formyl-H₄PtG₃. The triglutamate derivatives are pteroyl-γ-glutamyl-γ-glutamylglutamates (Boothe *et al.* 1948).

Growth of organisms and preparation of enzyme-containing extracts. *Escherichia coli* PA15, an auxotroph requiring serine or glycine for growth, was used throughout this work. Methods of maintenance, growth and subsequent preparation of ultrasonic extracts were as described by Guest *et al.* (1960). The extracts were dialysed against potassium phosphate buffer (0.05M; pH 7.8) and treated immediately before use with Dowex 1 (chloride form; X8; 100–200 mesh) resin by the method of Kisliuk & Woods (1960). The protein concentration of enzymic extracts was measured spectrophotometrically by the method of Layne (1957).

The extract of heated *E. coli* was prepared as described by Guest *et al.* (1960); an amount equivalent to 17 mg. dry wt. of organisms was used in tests for methionine synthesis.

Test for methionine synthesis. The basal reaction mixture contained (μmoles in a final volume of 2 ml.): potassium phosphate buffer, pH 7.8, 250; DL-homocysteine, 15; L-serine, 10; glucose, 20, or hexose diphosphate, 15; MgSO₄, 10; ATP, 10; DPN, 1; pyridoxal phosphate, 0.5. In later experiments FMN, 0.35, was added and also TPN, 0.1, when dihydrofolates (though not tetrahydrofolates) were present. When the formaldehyde derivative of H₄PtG, N⁵,N¹⁰-methyleneH₄PtG, was used as donor of the C₁ unit the amount of DL-homocysteine was decreased to 5 μmoles and pyridoxal phosphate was omitted (Kisliuk & Woods, 1960). The quantity of enzyme-containing extract generally used contained 10 mg. of protein. The complete reaction mixtures were incubated at 37° for 1.5–4 hr. in an atmosphere of H₂. The methionine formed was assayed micro-biologically with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 by the method described by Guest *et al.* (1960). Presence in the basal growth medium of aminopterin and amethopterin, at concentrations equivalent to those in the samples of reaction mixtures to be assayed, had no effect on the response of the organism to methionine. The synthesis of methionine is expressed throughout as μm-moles of L-methionine formed/mg. of protein.

Serine transhydroxymethylase. The basal reaction mixture for assaying the synthesis and breakdown of serine was the same as that used for testing the formation of methionine, except that L-serine, DL-homocysteine and FMN were omitted and pyridoxal phosphate was increased to 1 μmole. The reaction mixture also contained (μmoles in 2 ml.): H₄PtG, 5 (or H₂PtG, 5, plus TPN, 0.1); the substrates for serine formation were glycine, 10, and formaldehyde, 5; L-serine, 5, was used in studies of its catabolism. The reaction was started by adding dialysed and Dowex 1-treated ultrasonic extract equivalent to 10 mg. of protein and was carried out in Thunberg tubes under an atmosphere of H₂ at 37°. Trichloroacetic acid (0.8 ml. of 15%, w/v) was added to stop the reaction and the precipitate

was removed by centrifuging. Samples of the supernatant fluid (1.5 ml.) were assayed for serine by the method of Kisliuk & Woods (1960). The results are expressed as μmoles of serine formed or destroyed in a period during which the rate of change of serine concentration was constant. The value obtained for a control containing all components of the reaction mixture, but stopped at zero time, was deducted.

Dihydrofolate reductase. The assay of this enzyme was based on the method of Osborn & Huenekens (1958). The reaction mixture (0.4 ml. in a cuvette having an optical path of 1 cm.) contained dialysed and Dowex 1-treated extract equivalent to 0.2 mg. of protein and (μmoles): phosphate buffer, pH 7.4, 16; 2-mercaptoethanol, 4; dihydrofolate, 0.025; reduced TPN or reduced DPN, 0.04. The reaction was followed by the change in extinction at 340 mμ in the Unicam spectrophotometer SP. 500 or the Cary recording spectrophotometer model 14M-50. After subtraction of the value for the endogenous oxidation of reduced pyridine nucleotide the change in extinction is the sum of changes in spectrum caused by the oxidation of the pyridine nucleotide and the reduction of the folate; the relative contributions of each are approx. 55% and 45% respectively.

Chemicals. H₄PtG was prepared from PtG (British Drug Houses Ltd., Poole, Dorset) by catalytic hydrogenation in acetic acid with platonic oxide (Adams catalyst) by the method of O'Dell, Vandenberg, Bloom & Piffner (1947); the product was stored as a solid under H₂. H₄PtG₃ was prepared by a similar catalytic hydrogenation of a solution of PtG₃ (a gift from Dr E. L. R. Stokstad) in phosphate buffer (25 mM, pH 7.0) by the method of Jones *et al.* (1961).

H₂PtG and H₂PtG₃ were prepared by the method of Futterman (1957) and both were stored as solids under H₂. These compounds are probably 7,8-dihydropteroyl derivatives having no asymmetric centre (Perault & Pullman, 1960; Rabinowitz, 1960); this is supported by a comparison of their biological activities with the chemically prepared tetrahydropteroyl derivatives which are racemates (Osborn & Huenekens, 1958; Peters & Greenberg, 1958). Aminopterin (4-aminoPtG) and amethopterin (N¹⁰-methyl-4-aminoPtG) were kindly supplied by Dr H. P. Broquist.

Crystalline cobalamin was obtained from British Drug Houses Ltd.; it was 88% pure by spectrophotometric assay. ATP, FMN, DPN, TPN, reduced DPN and reduced TPN were products of Sigma Chemical Co., St Louis, Mo., U.S.A. Hexose diphosphate (British Drug Houses Ltd.), pyridoxal phosphate (Roche Products Ltd., Welwyn Garden City, Herts.) and DL-homocysteine (Mann Research Laboratories Inc., New York, U.S.A.) were also commercial specimens.

RESULTS

Dihydrofolates as cofactors in the overall synthesis of methionine

Both H₂PtG and H₂PtG₃ resembled the corresponding tetrahydro-derivatives in their ability to promote the synthesis of methionine (Table 1). Cobalamin was essential for the activity of the monoglutamates whereas that of the triglutamates was independent of this vitamin; under the required conditions the triglutamates were about

Table 1. *Dihydrofolates as cofactors for the synthesis of methionine by ultrasonic extracts of Escherichia coli*

The basal reaction mixture also contained reduced PtG (1 μ mole) or reduced PtG₃ (0.1 μ mole) and cobalamin (0.65 μ m-mole) when present. Incubation was for 2 hr.

Folic acid cofactor	L-Methionine formed (μ m-moles/mg. of protein)	
	Cobalamin absent	Cobalamin present
None	4	6
H ₂ PtG	3	62
H ₄ PtG	2	64
H ₂ PtG ₃	105	112
H ₄ PtG ₃	106	108

Table 2. *Effect of dihydrofolates on the synthesis of methionine with an extract of heated Escherichia coli as source of the folic acid cofactor*

The basal reaction mixture also contained extract of heated *E. coli* equivalent to 17 mg. dry wt. of organisms and, when present, reduced folates (1 μ mole) and cobalamin (0.65 μ m-mole). Incubation was for 2 hr.

Folate added in addition to the extract of heated <i>E. coli</i>	L-Methionine formed (μ m-moles/mg. of protein)	
	Cobalamin absent	Cobalamin present
None	64	65
H ₂ PtG	10	68
H ₄ PtG	3	63
H ₂ PtG ₃	100	115
H ₄ PtG ₃	105	105

Fig. 1. Activity of reduced folates as cofactors for the synthesis of methionine by ultrasonic extracts of *Escherichia coli* PA 15. The basal reaction mixture also contained: (a) H₂PtG or H₄PtG and, in either case, cobalamin (1 μ m-mole); (b) H₂PtG₃ or H₄PtG₃. *S*, Concentration of reduced folate (μ mole/ml.); *V*, rate of methionine formation (μ mole of L-methionine formed/10 mg. of protein/2 hr.). ●, Tetrahydro-derivative; ○, dihydro-derivative.

ten times as active as the monoglutamates. H₂PtG also resembled H₄PtG in that it inhibited the synthesis of methionine when the folic acid cofactor was supplied by an extract of heated organisms (in the absence of added cobalamin); in both cases the inhibition was overcome by addition of cobalamin (Table 2). H₄PtG₃ and H₂PtG₃, however, had no inhibitory effect under these conditions (Table 2).

The dihydrofolates were almost twice as active as the corresponding tetrahydrofolates (Fig. 1); the Michaelis constant (*K_m*) values for H₂PtG and H₄PtG were 113 and 203 μ M respectively, and those for H₂PtG₃ and H₄PtG₃ were 7.9 and 14.8 μ M respectively. Such relative figures were obtained with several batches of ultrasonic extract, though the *K_m* values for each pair of mono- or tri-glutamates varied by $\pm 25\%$. The approximately double activity of the dihydro-compounds supports the conclusion that the tetrahydro-derivatives contain 50% of a biologically inactive stereoisomer. The concentration of H₂PtG (14 μ M) causing 50% inhibition of methionine formation when the extract of heated *E. coli* was the source of cofactor was also significantly lower than that (20 μ M) for H₄PtG.

Later in this work a flavin coenzyme was found to be necessary for optimum synthesis of methionine (Table 3); this agrees with the observations of Hatch *et al.* (1959). Previously no such requirement was demonstrable in this Laboratory; a more effective removal of anionic cofactors with a new batch of Dowex 1 resin probably accounts for the changed result. With H₂PtG these same extracts were relatively less active than with H₄PtG for the synthesis of methionine even in the presence of FMN. However, the further addition of TPN raised methionine synthesis almost to the value obtained with H₄PtG, although it decreased the activity with the latter as C₁-carrier (Table 3); it was concluded that TPN was specifically concerned with the utilization of H₂PtG.

Table 3. *Effect of flavin mononucleotide and triphosphopyridine nucleotide on the synthesis of methionine*

The basal reaction mixture also contained H₂PtG or H₄PtG (1 μ mole), cobalamin (0.65 μ m-mole) and, when present, FMN (0.3 μ mole) and TPN (0.1 μ mole). Incubation was for 2.5 hr.

Additions to basal reaction mixture	L-Methionine formed (μ m-moles/mg. of protein)	
	H ₄ PtG present	H ₂ PtG present
None	63	28
FMN	82	40
TPN	43	45
FMN + TPN	44	74

Table 4. Effect of aminopterin and amethopterin on the synthesis of methionine

The basal reaction mixture also contained either (a) extract of heated *E. coli* equivalent to 17 mg. dry wt. of organisms, incubated for 2 hr., or (b) reduced PtG (1 μ mole) and cobalamin (65 μ moles), incubated for 4 hr., or (c) reduced PtG₃ (0.1 μ mole), incubated for 2 hr.

Source of the folic acid cofactor	L-Methionine formed (μ m-moles/mg. of protein)							
	Aminopterin (μ M)				Amethopterin (μ M)			
	0	0.05	0.5	5.0	0	0.005	0.05	0.5
(a) Extract of heated <i>E. coli</i>	55	53	57	55	92	95	90	92
(b) H ₂ PtG	60	34	8	4	66	56	33	9
H ₄ PtG	60	56	58	62	70	72	72	74
(c) H ₂ PtG ₃	100	62	15	5				
H ₄ PtG ₃	92	95	99	98				

Fig. 2. Effect of aminopterin on methionine formation by ultrasonic extracts of *Escherichia coli* PA15 with dihydropteroylmonoglutarate as cofactor. *S*, Concentration of H₂PtG (μ moles/ml.); *V*, rate of methionine formation (μ moles of L-methionine formed/10 mg. of protein/2.5 hr.). Concentration (μ mM) of aminopterin: $\circ$, nil; $\bullet$, 15; $\bullet$, 50; $\bullet$, 250.

Effect of analogues of folic acid on the cofactor activity of reduced folates. Aminopterin and amethopterin had no effect on the synthesis of methionine when either extract of heated organisms or H₄PtG was the source of the cofactor (Table 4). However, with H₂PtG, methionine formation was reduced almost to insignificant values by both analogues. Similarly, aminopterin caused 95% inhibition of methionine formation with H₂PtG₃, whereas the activity with H₄PtG₃ was unaffected (Table 4). Higher concentrations of aminopterin or amethopterin could not be used because they affected the growth of *Leuconostoc mesenteroides* P 60 in the assay of methionine. The inhibition was examined quantitatively by the method of Lineweaver & Burk (1934) and found to be non-

Table 5. Effect of aminopterin on the synthesis of methionine in the presence of catalytic levels of tetrahydropteroylmonoglutarate

The basal reaction mixture contained an increased amount (20 mg. of protein) of Dowex 1-treated ultrasonic extract and also cobalamin (1 μ m-mole). Time of incubation, 4 hr.

H ₄ PtG (μ mole)	L-Methionine formed (μ moles)		L-Methionine formed (moles/mole of folate added)
	Aminopterin absent	Aminopterin (5 μ M) present	
0.1	0.64	0.60	6.4
0.2	0.99	0.93	5.0
0.4	1.42	1.37	3.6

competitive, as shown for H₂PtG and aminopterin in Fig. 2; *K_i* for aminopterin was 66 μ mM. With H₂PtG₃, *K_i* for aminopterin was 88 μ mM.

Since the only clearly established action of aminopterin and amethopterin with isolated enzymes is the inhibition of dihydrofolate reductase, these results suggest that dihydrofolates must be converted into tetrahydrofolates before they can activate the synthesis of methionine.

Effect of analogues of folic acid when folate is used at catalytic levels. If the amount of ultrasonic extract used for testing the synthesis of methionine was doubled, H₄PtG was clearly shown to be catalytic in action (Table 5). Depending upon the concentration of folate used, between 3 and 6 molecules of methionine were formed per molecule of H₄PtG present. Furthermore, this ratio should probably be doubled to between 6 and 12 because the H₄PtG used was a racemate and one isomer is almost certainly inactive. Aminopterin (5 μ M) had no significant effect under these conditions (Table 5), although with H₂PtG (0.5 μ mole) the synthesis of methionine (1.5 μ moles/4 hr.) was inhibited by 98%. The catalytic action of the folate was even more clear-cut when H₄PtG₃ was used: between 7 and 30 molecules of methionine

were formed per molecule of H_4PtG_3 over the range studied (Fig. 3). Aminopterin again had virtually no effect on methionine formation (Fig. 3) at a concentration which caused 95 % inhibition with H_2PtG_3 (Table 4). If the primary action of aminopterin is to inhibit the activity of dihydrofolate reductase, these results, in conjunction with those of the preceding section, indicate that dihydrofolate is not formed as a free intermediate in the synthesis of the methyl group of methionine.

Effect of folic acid analogues on dihydrofolate reductase. The cell-free extracts used in the above-mentioned studies were found to contain the enzyme dihydrofolate reductase, which catalysed the reduction of both H_2PtG and H_2PtG_3 to the corresponding tetrahydro-derivatives (Fig. 4); the enzyme was pyridine nucleotide-linked. The specific activity of typical extracts (expressed in terms of the disappearance of reduced TPN caused by the reduction of dihydrofolate) was approximately 75–90 $\mu\text{m-moles/mg. of protein/hr.}$ Compared with the rates normally obtained for the formation of methionine under the several conditions described (12–50 $\mu\text{m-moles/mg. of protein/hr.}$) the reductase was two to six times as active, despite being assayed at a temperature 17° lower than is used for estimating the formation of methionine. Thus if the utilization of H_2PtG and H_2PtG_3 depends upon their conversion into H_4PtG and H_4PtG_3 , the reductase would probably be capable of catalysing this reaction at a rate which would not limit the formation of methionine. K_m values for both H_2PtG and H_2PtG_3 in the reductase reaction varied between 0.8 and 3.0 μM for different batches of the extract. The enzyme was most readily demonstrated with reduced TPN as the

reducing agent because a high endogenous oxidation of reduced DPN masked any activity obtained with the latter. The endogenous oxidation of reduced DPN was decreased by 45 % saturation of the extract with ammonium sulphate; the dialysed supernatant fraction promoted the oxidation of reduced DPN in the presence of H_2PtG although the rate was less than 10 % of the rate of oxidation of reduced TPN by the same fraction and was only just detectable. It was concluded that the enzyme is primarily TPN-linked. The enzyme was completely inhibited by aminopterin at concentrations which inhibited methionine formation (Table 6);

Fig. 4. Dihydrofolate reductase in ultrasonic extracts of *Escherichia coli* PA15. Reaction mixture (final volume, 0.4 ml.) was as described in Methods and Materials section. Incubation was at room temperature (20°) in spectrophotometer cuvettes having an optical path of 1 cm. ---, Reduced TPN alone; - · -, H_2PtG alone; —, H_2PtG plus reduced TPN. Similar results were obtained with H_2PtG_3 .

Fig. 3. Effect of aminopterin on the synthesis of methionine with tetrahydropteroyltriL-glutamate as the cofactor. Reaction mixture plus H_4PtG_3 as shown and with aminopterin (5 μM) absent (●) or present (○). ---, Ratio methionine (moles) formed/ H_4PtG_3 (moles) added.

Table 6. Inhibition of dihydrofolate reductase by aminopterin

Reaction mixture was as described in the Methods and Materials section with dihydrofolate (60 μM). Incubated at room temperature (20°).

Aminopterin concn. (μM)	Rate of change of E at 340 $\text{m}\mu$ (unit/hr.)*	
	H_2PtG present	H_2PtG_3 present
0	0.37	0.35
0.0006	0.32	0.33
0.006	0.26	0.28
0.06	0.02	0.01
0.6	0	0
6.0	0	0

* Endogenous oxidation of reduced TPN was 0.03 in all cases and has been deducted.

the weak activity of the enzyme in the crude extracts precluded accurate measurements of the kinetics of this inhibition.

Formaldehyde as the donor of the C₁ unit for the synthesis of methionine. When serine is the source of the methyl group of methionine, the methylation of homocysteine by *E. coli* may be considered in two stages: (a) the conversion of serine into glycine and a C₁ unit by the action of serine transhydroxymethylase and (b) the conversion of the C₁ unit into the methyl group of methionine. Both these reactions probably require tetrahydrofolate, the dihydro-derivatives being inactive in each case. To investigate the nature of the requirement for folate in the second stage of the overall reaction formaldehyde was used as the source of the C₁ unit. Methionine synthesis occurred when N⁵N¹⁰-methyleneH₄PtG (provided as an equimolar mixture of formaldehyde and H₄PtG) replaced serine; aminopterin did not inhibit (Table 7). When, however, an equimolar mixture of formaldehyde and H₂PtG (which by analogy should yield N⁵N¹⁰-methyleneH₂PtG) was used, virtually no methionine was formed even when aminopterin

was absent. This result is almost certainly due to the low affinity of H₂PtG for formaldehyde compared with that of H₄PtG (Blakley, 1959). Consequently, spontaneous condensation of formaldehyde with homocysteine to form 1,3-thiazane-4-carboxylic acid would occur and thus reduce the concentration of both these essential reactants. By using Thunberg tubes with two side arms it was possible to show that a first incubation of H₂PtG in the reaction mixture without formaldehyde produced a compound (probably H₄PtG) which permitted the utilization of formaldehyde (Table 8). This effect was prevented by the addition of aminopterin during the first incubation but not later. These results indicate that the tetrahydro-derivative is essential for the second stage of the overall reaction of methionine formation and that the dihydro-derivative is not directly active.

Dihydrofolates and the metabolism of serine

H₂PtG has been found to activate crude preparations of serine transhydroxymethylase isolated from rabbit liver, although it was inactive with the purified enzyme (Blakley, 1957, 1959). A strict requirement for H₄PtG as the cofactor for serine transhydroxymethylase and thymidylate synthetase has also been shown by Greenberg, Malkin & Nath (1960); Rabinowitz (1960) has suggested that systems responding to more oxidized forms of folic acid probably contain enzymes for their reduction. The formation of serine from glycine and formaldehyde by dialysed and Dowex 1-treated ultrasonic extracts of *E. coli* PA 15 was examined under conditions similar to those used for testing the formation of methionine (Table 9). A folic acid cofactor was essential for the formation of serine and this could be supplied by either H₂PtG or H₄PtG. However, the action of H₂PtG was inhibited by aminopterin and was strictly dependent upon the presence of TPN and a glycolytic system (glucose or hexose diphosphate, ATP, magnesium sulphate and DPN). With H₄PtG, on the other hand, serine

Table 7. *Effect of aminopterin on the synthesis of methionine when formaldehyde is the source of the C₁ unit*

L-Serine and pyridoxal phosphate were omitted from the basal reaction mixture and DL-homocysteine was decreased to 5 μmoles; cobalamin (65 μμmoles) and substances indicated were added. Incubation was for 4 hr.

Source of the C ₁ unit and folic acid cofactor (μmoles of each)	L-Methionine formed (μm-moles/mg. of protein)	
	Aminopterin absent	Aminopterin (5 μM) present
Formaldehyde + { H ₂ PtG (1) H ₄ PtG (3)	3 2	4 3
Formaldehyde + { H ₄ PtG (1) H ₄ PtG (3)	19 51	20 51

Table 8. *Dihydropteroylglutamate and the utilization of formaldehyde for the synthesis of methionine*

L-Serine, DL-homocysteine and pyridoxal phosphate were omitted from the basal reaction mixture and cobalamin (65 μμmoles) was added. H₂PtG (3 μmoles), formaldehyde (2.5 μmoles), DL-homocysteine (4 μmoles) and aminopterin (10 μmoles; final concn. 5 μM) were added as indicated. The reaction vessel was a Thunberg tube with two side arms filled with H₂.

Additions to the basal reaction mixture during			L-Methionine formed (μm-moles/mg. of protein)
First incubation (60 min.)	Second incubation (2 min.)	Third incubation (120 min.)	
None	H ₂ PtG + formaldehyde	Homocysteine	5
H ₂ PtG	Formaldehyde	Homocysteine	16
H ₂ PtG + aminopterin	Formaldehyde	Homocysteine	4
H ₂ PtG	Formaldehyde + aminopterin	Homocysteine	15

Table 9. *Effect of dihydro- and tetrahydro-pteroylmonoglutamate on the formation of serine from glycine and formaldehyde*

The basal reaction mixture (see Methods and Materials section) also contained glycine (10 μ moles), formaldehyde (5 μ moles) and either (a) H_2 PtG (5 μ moles) plus TPN (0.1 μ mole) or (b) H_4 PtG (5 μ moles). Time of incubation: (a) 20 min. (b) 10 min.

		Concn. of aminopterin (μ M)					
		0	0.005	0.05	0.5	5.0	50
Folic acid cofactor	Modifications to the reaction mixture	Serine formed (μ moles)					
None	None	0.3	—	—	—	—	0.2
(a) H_2 PtG	None	2.3	2.0	1.25	0.9	0.1	0.3
	Substrate and cofactors* for glycolysis omitted	0.3	—	—	—	—	—
	TPN omitted	0.4	—	—	—	—	—
(b) H_4 PtG	None	2.1	2.05	1.75	1.7	1.8	2.0
	Substrate and cofactors* for glycolysis omitted	1.9	—	—	—	—	—
	TPN omitted	1.9	—	—	—	—	—

* Glucose (or hexose diphosphate), ATP, $MgSO_4$ and DPN.

Table 10. *Effect of dihydro- and tetrahydro-pteroylmonoglutamate on the breakdown of serine*

The basal reaction mixture (see Methods and Materials section) was supplemented with L-serine (5 μ moles) and either (a) H_2 PtG (5 μ moles) plus TPN (0.1 μ mole) or (b) H_4 PtG (5 μ moles). The final concentration of aminopterin was 50 μ M, when present. Incubation was for 10 min. in both cases.

Folic acid cofactor	Modification of the reaction mixture	Serine destroyed (μ moles)
None	None	0.2
(a) H_2 PtG	None	1.1
	Aminopterin added	0.15
	Substrate and cofactors* for glycolysis omitted	0.3
	TPN omitted	0.15
(b) H_4 PtG	None	2.0
	Aminopterin added	2.1
	Substrate and cofactors* for glycolysis omitted	2.2
	TPN added	2.3

* Glucose (or hexose diphosphate), ATP, $MgSO_4$ and DPN.

formation was not greatly affected by aminopterin, it was independent of the glycolytic system and was little changed by the addition of TPN. The reverse reaction (degradation of serine to glycine) was also examined and similar results were obtained (Table 10). The action of H_2 PtG was inhibited by aminopterin and the presence of the glycolytic system and TPN were essential, whereas with H_4 PtG activity was not affected by aminopterin, TPN or absence of the substrate and cofactors for glycolysis. It was, however, noticed that with

H_2 PtG the amount of serine destroyed at equilibrium was only 25–30 % of that with H_4 PtG. Cobalamin has already been shown to have no effect on either of these reactions (Kisliuk & Woods, 1960; Guest, 1960).

Analogues of folic acid and the inhibition of methionine synthesis by reduced pteroylmonoglutamate

Both aminopterin and amethopterin largely prevented the inhibition of methionine formation caused by H_2 PtG when extract of heated *E. coli* supplied the folic acid cofactor in the absence of cobalamin (Table 11). The similar inhibition caused by H_4 PtG was unaffected by these analogues, suggesting that H_2 PtG exerts its inhibitory activity as well as its cofactor activity only after conversion into H_4 PtG. Other reduced pteroylmonoglutamates (N^5 - and N^{10} -formyl H_4 PtG and N^5N^{10} -methenyl H_4 PtG), which have a limited capacity to serve as cofactors in this system when cobalamin is present, also inhibit the action of the cofactors present in the extract of heated *E. coli* in the absence of added cobalamin (Guest, 1960). However, such activities may again be due to their conversion into unsubstituted H_4 PtG.

DISCUSSION

The dihydro-derivatives of both PtG and PtG₃ resembled the corresponding tetrahydro-derivatives in their action as cofactors and inhibitors of the synthesis of methionine by cell-free extracts of *E. coli* PA 15. For several reasons it is concluded that the dihydro-derivatives are active only after further reduction to the tetrahydro-compounds. First, analogues of folic acid inhibited the action of

Table 11. *Effect of folic acid analogues on the inhibition of methionine synthesis by reduced pteroylmonoglutamates*

The basal reaction mixture also contained extract of heated *E. coli* equivalent to 17 mg. dry wt. of organisms and (when added) reduced PtG (1 μ mole). Incubation was for 3 hr.

Folic acid added	L-Methionine formed (μ m-moles/mg. of protein)					
	Aminopterin (μ M)			Amethopterin (μ M)		
	0	0.5	5.0	0	0.05	0.5
None	74	72	74	92	90	90
H ₂ PtG	12	39	60	12	32	65
H ₄ PtG	3	4	3	6	6	6

the dihydro-compounds (but not of the tetrahydro-compounds) both in methionine formation and in the synthesis and breakdown of serine. Secondly, the presence of a glycolytic system was essential for the utilization of H₂PtG in the synthesis and breakdown of serine, presumably for the generation of reduced pyridine nucleotides to convert it into H₄PtG. Thirdly, with H₂PtG the enzymic extract produced a compound (probably H₄PtG) which permitted the utilization of formaldehyde as a source of the methyl group of methionine. The formation, but not the subsequent activity, of this intermediate was sensitive to aminopterin. Finally, the extracts contained a dihydrofolate reductase which was primarily a TPN-linked enzyme and was strongly inhibited by aminopterin. This observation also explains the fact that with some batches of the enzymic extract TPN was essential for optimum synthesis of methionine and for the synthesis and breakdown of serine when H₂PtG was the cofactor, whereas TPN was unnecessary or even inhibitory with H₄PtG.

If it is accepted that aminopterin inhibits the conversion of H₂PtG into H₄PtG it is probable that only the natural isomer of the latter is responsible for inhibiting the cofactor activity of extract of heated *E. coli* and of H₄PtG₃ in the absence of cobalamin. Only the natural stereoisomer of H₄PtG would be formed enzymically from H₂PtG (which has no asymmetric centre), thus eliminating the possibility that the inhibition is due to the unnatural isomer present in the chemically prepared racemate. Good, though not conclusive, evidence that the inhibition was not caused by an impurity or decomposition product in the racemic compound had already been obtained by the use of highly purified H₄PtG (Guest, 1960). It is therefore concluded that in the absence of cobalamin H₄PtG acts as an inhibitory analogue of the folic acid coenzymes present in the extract of heated *E. coli*; these probably include N⁵-formyl-H₄PtG₃.

Alternative hypotheses for the formation of the methyl group of methionine are shown in Fig. 5. In the first (*A*) the reduction of the hydroxymethyl group (derived from serine or formaldehyde) is coupled to the oxidation of tetrahydrofolate with liberation of the dihydro-derivative and its subsequent reconversion into the tetrahydro-compound by the action of dihydrofolate reductase in the presence of reduced TPN. There is evidence for such a mechanism in the synthesis of the methyl groups of thymidylate by extracts of rat thymus gland, *Streptococcus faecalis* R and *E. coli* (Humphreys & Greenberg, 1958; McDougall & Blakley, 1961; Wahba & Friedkin, 1961), choline by rat-liver homogenates (Venkataraman & Greenberg, 1958; Peters & Greenberg, 1958) and methionine by pig-liver enzymes (Wilmanns *et al.* 1960). On the second hypothesis (Fig. 5, *B*) some other agent, XH₂ (possibly reduced pyridine nucleotide or a reduced flavin nucleotide), reduces the C₁ unit and the folate is liberated directly as the tetrahydro-derivative.

Under the conditions of the present experiments the action of tetrahydrofolate (either H₄PtG or H₄PtG₃) was demonstrably catalytic, yet methionine synthesis was unaffected by concentrations of aminopterin which inhibited (by over 95 %) both the activity of dihydrofolate reductase and the synthesis of methionine with dihydrofolate (either H₄PtG or H₂PtG₃) as cofactor. Failure to demonstrate a stoicheiometric relationship between methionine and available reducing power (because of the high endogenous oxidation of reduced pyridine nucleotides by the ultrasonic extract) prevented further confirmation of the deductions made from the results obtained with the folate analogues. It is concluded provisionally that the reduction of the hydroxymethyl group in the synthesis of methionine by *E. coli* is not accomplished by tetrahydrofolate itself acting as the immediate reducing agent with the consequent liberation of free dihydrofolate. The reaction would thus differ from the apparently analogous cases of the formation of the methyl groups of thymidylate, choline and also methionine in animal tissues. A further difference between the reaction studied here and these other reactions is indicated by the fact that H₄PtG will serve as the cofactor for thymidylate synthesis by extracts of *E. coli* PA15 in the absence of cobalamin (Birnie & Crosbie, 1958), whereas this vitamin is essential for the synthesis of methionine under similar conditions. Furthermore, no requirement for cobalamin has been found in the formation of methionine with H₄PtG as the cofactor with enzymes from mammalian liver (Wilmanns *et al.* 1960; Stevens & Sakami, 1959; Nakao & Greenberg, 1958).

Although the present organism has been shown to contain a dihydrofolate reductase which is

sensitive to aminopterin, there remains a formal possibility that H_2PtG or H_4PtG_3 may be reduced to H_4PtG or H_4PtG_3 by some other reaction which is less sensitive to the analogue; this is conceivable, for example, if isomers of H_2PtG other than 7,8-dihydroPtG are intermediates. Under such circumstances mechanism A (Fig. 5) would not be eliminated, though it would then be necessary to postulate an inhibitory function for aminopterin other than in the conversion of dihydrofolate into tetrahydrofolate when dihydrofolate is the co-factor, e.g. an inhibition specific to a hypothetical direct use of dihydrofolate as cofactor. Mechanism B is therefore presented as the most probable

working hypothesis for methionine synthesis in *E. coli* PA 15, though confirmation must await the isolation of purified enzymes for this reaction.

Possible intermediates in the synthesis of methionine by *E. coli* include a complex of tetrahydrofolate and homocysteine linked by a methylene bridge which is reductively cleaved (Kisliuk & Woods, 1960), or N^5 -methyltetrahydrofolate. N^{10} -Methyl H_4PtG prepared from N^{10} -methylPtG (a gift from Dr E. L. R. Stokstad) did not serve as the source of the methyl group of methionine (Guest, 1960). Wilmanns *et al.* (1960) have proposed the structure N^5 -methyl-7,8-dihydropteroylmonoglutamate for an isolated intermediate

Fig. 5. Possible mechanisms for the reduction of the C_1 unit in the formation of the methyl group of methionine.

formed from N^5N^{10} -methylene H_4 PtG by enzymes concerned with the synthesis of methionine in mammalian liver. Larrabee & Buchanan (1961) have also isolated a methylated reduced folate as an intermediate in methionine formation in *E. coli*. In view of the present results a N^5 -methyltetrahydrofolate would be the most satisfactory structure for such an intermediate; this could be formed by direct reduction of N^5N^{10} -methylene-tetrahydrofolate. If N^5 -methyl-dihydrofolate were an intermediate it would be necessary to postulate either that there is reductive transfer of the methyl group, resulting in the liberation of tetrahydrofolate, or that the methyl-dihydro-derivative is further reduced to N^5 -methyltetrahydrofolate by a reaction not inhibited by aminopterin.

SUMMARY

1. Dihydropteroylmonoglutamate and dihydropteroyltriglutamate resembled the corresponding tetrahydro-derivatives in their ability to serve as cofactors for the synthesis of methionine from serine and homocysteine by extracts of *Escherichia coli* PA15. Cobalamin was a necessary component of the reaction mixture only if the pteroylmonoglutamates were the cofactors for C_1 transfer.

2. A flavin coenzyme was required for best synthesis of methionine with some preparations of the extract and, with dihydrofolates as cofactor, triphosphopyridine nucleotide was also necessary.

3. Dihydropteroylmonoglutamate also resembled the tetrahydro-derivative in inhibiting methionine formation when the folic acid cofactor was supplied by an extract of heated *E. coli*; the inhibition was overcome by cobalamin.

4. Aminopterin and amethopterin prevented both the cofactor and inhibitory activities of the dihydrofolates; the activities of the tetrahydrofolates, even when present only in catalytic quantities, were unaffected by these analogues.

5. In addition to the above evidence the characterization of an aminopterin-sensitive dihydrofolate reductase in these bacterial extracts supported the view that the dihydro-compounds were active only after conversion into the tetrahydro-compounds.

6. Aminopterin also prevented the utilization of formaldehyde as a source of the methyl group of methionine when dihydropteroylmonoglutamate, though not tetrahydropteroylmonoglutamate, was the cofactor for the C_1 transfer.

7. Under conditions similar to those used for the synthesis of methionine both dihydro- and tetrahydro-pteroylmonoglutamate acted as cofactors for serine transhydroxymethylase; aminopterin inhibited the activity only of the dihydro-derivative.

8. It is concluded provisionally that dihydropteroylglutamate is not liberated during the reduction of the hydroxymethyl group of serine to the methyl group of methionine by *E. coli*.

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REFERENCES

- Birnie, G. D. & Crosbie, G. W. (1958). *Biochem. J.* **69**, 1r.
 Blakley, R. L. (1957). *Biochem. J.* **65**, 342.
 Blakley, R. L. (1959). *Biochem. J.* **72**, 707.
 Boothe, J. H., Mowat, J. H., Hutchings, B. L., Angier, R. B., Waller, C. W., Stokstad, E. L. R., Semb, A. L., Gazzola, A. L. & SubbaRow, Y. (1948). *J. Amer. chem. Soc.* **70**, 1099.
 Friedkin, M. (1959). *Fed. Proc.* **18**, 230.
 Futterman, S. (1957). *J. biol. Chem.* **228**, 1031.
 Greenberg, D. M., Malkin, L. I. & Nath, R. (1960). *Biochem. biophys. Res. Commun.* **3**, 603.
 Guest, J. R. (1960). D.Phil. Thesis: Oxford University.
 Guest, J. R., Helleiner, C. W., Cross, M. J. & Woods, D. D. (1960). *Biochem. J.* **76**, 396.
 Guest, J. R. & Woods, D. D. (1960). *Biochem. J.* **77**, 422.
 Hatch, F. T., Takeyama, S., Cathou, R. E., Larrabee, A. R. & Buchanan, J. M. (1959). *J. Amer. chem. Soc.* **81**, 6525.
 Humphreys, G. K. & Greenberg, D. M. (1958). *Arch. Biochem. Biophys.* **78**, 275.
 Jones, K. M., Guest, J. R. & Woods, D. D. (1961). *Biochem. J.* **79**, 566.
 Kisliuk, R. L. & Woods, D. D. (1960). *Biochem. J.* **75**, 467.
 Larrabee, A. R. & Buchanan, J. M. (1961). *Fed. Proc.* **20**, 9.
 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.
 McDougall, B. M. & Blakley, R. L. (1961). *J. biol. Chem.* **236**, 832.
 Nakao, A. & Greenberg, D. M. (1958). *J. biol. Chem.* **230**, 603.
 O'Dell, B. L., Vandenbelt, J. M., Bloom, E. S. & Piffner, J. J. (1947). *J. Amer. chem. Soc.* **69**, 250.
 Osborn, M. J., Freeman, M. & Huennekens, F. M. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 429.
 Osborn, M. J. & Huennekens, F. M. (1958). *J. biol. Chem.* **233**, 969.
 Perault, A.-M. & Pullman, B. (1960). *Biochim. biophys. Acta*, **44**, 251.
 Peters, J. M. & Greenberg, D. M. (1958). *J. Amer. chem. Soc.* **80**, 6679.
 Rabinowitz, J. C. (1960). In *The Enzymes*, vol. 2, p. 185. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
 Stevens, A. & Sakami, W. (1959). *J. biol. Chem.* **234**, 2063.
 Szulmajster, J. & Woods, D. D. (1960). *Biochem. J.* **75**, 3.
 Venkataraman, R. & Greenberg, D. M. (1958). *J. Amer. chem. Soc.* **80**, 2025.
 Wahba, A. J. & Friedkin, M. (1961). *J. biol. Chem.* **236**, PC11.
 Wilmanns, W., Rücker, B. & Jaenicke, L. (1960). *Hoppe-Seyl. Z.* **322**, 283.