The Interaction of Folic Acid Derivatives in the Methylation of Homocysteine

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1. The cobalamin-independent synthesis of methionine from serine and homocysteine by ultrasonic extracts of *E. coli* with tetrahydropteroyltriglutamate as cofactor was inhibited competitively by tetrahydropteroylmonoglutamate and derivatives which were readily converted into this compound. 2. The potency of these inhibitors was directly related to their ability to function as cofactors or substrates in the alternative, cobalamin-dependent mechanism for homocysteine methylation. 3. The cobalamin-dependent and -independent mechanisms of homocysteine methylation were both inhibited by reduced derivatives of aminopterin in a similar manner. 4. It was tentatively concluded that the inhibition was due to a competitive interaction between the folates for N^5N^{10} -methylenetetrahydrofolate reductase.

Two mechanisms have been established for the methylation of homocysteine in *Escherichia coli* (Guest & Woods, 1962a; Foster, Tejerina, Guest & Woods, 1964; Woods, Foster & Guest, 1965). The formation of N^5N^{10} -methylenetetrahydrofolate from serine by serine hydroxymethyltransferase and its subsequent reduction to the N^5 -methyl derivative by N^5N^{10} -methylenetetrahydrofolate reductase are common to both pathways. The methylation of homocysteine is then catalysed either by N^5 -methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, which is specific for N^5 -methyl H_4PtG_3 ,† or by a cobalamin-containing methyltransferase (cobalaminmethyltransferase) which utilizes both N^5 -methyl H_4PtG and N^5 -methyl H_4PtG_3 as substrates (Guest, Foster & Woods, 1964a; Guest, Friedman, Foster, Tejerina & Woods, 1964c). Organisms grown in the presence of cobalamin contain cobalaminmethyltransferase (Kisliuk & Woods, 1960), and organisms grown without added cobalamin possess the corresponding apoenzyme from which the active holoenzyme can be generated in cell-free extracts by incubating with several derivatives of cobalamin under suitable conditions (Guest & Woods, 1960; Guest, Friedman, Dilworth & Woods, 1964b).

In the absence of cobalaminmethyltransferase, H_4PtG inhibits the enzymic formation of methionine

from serine and homocysteine when H_4PtG_3 or a heated extract of *E. coli* is supplied as source of conjugated folic acid coenzyme; this inhibition is overcome by the addition of cobalamin or cobalaminmethyltransferase (Kisliuk & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960). The competitive nature of this inhibition was later demonstrated by Jones, Guest & Woods (1961).

An examination of the established reactions leading to methionine (Scheme 1) indicates several possible mechanisms whereby the monoglutamate could inhibit the overall formation of methionine, in the absence of cobalaminmethyltransferase.

(1) Specific inhibition by derivatives of H_4PtG . Two of the three enzymes which comprise the triglutamate-specific pathway are also common to the cobalamin-dependent mechanism. The third, N^5 -methyl H_4PtG_3 methyltransferase, is unique to the first pathway and is therefore a possible site for inhibition. In this case the inhibitor could be H_4PtG itself or its N^5N^{10} -methylene and N^5 -methyl derivatives which may accumulate in the absence of cobalaminmethyltransferase. Alternatively, a feed-back mechanism could operate in which the accumulating N^5 -methyl derivative may interfere specifically with serine hydroxymethyltransferase, methylenetetrahydrofolate reductase, or both.

(2) Non-enzymic competition for one-carbon-atom units. In view of the non-enzymic dissociation of N^5N^{10} -methylenetetrahydrofolates into formaldehyde and tetrahydrofolate (Blakley, 1959), equilibration of the C_1 units between the mono- and tri-glutamate derivatives is possible. If H_4PtG has a greater capacity for binding formalde-

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† Abbreviations for members of the folic acid group are based on PtG for pteroylglutamate: H_4PtG , tetrahydropteroylglutamate; H_4PtG_3 , tetrahydropteroyltriglutamate; derivatives of these compounds are indicated as, e.g. N^5N^{10} -methylene H_4PtG_3 . Folate is reserved for general reference to the family of vitamin derivatives.

hyde than H_4PtG_3 , the monoglutamate could compete successfully for the available C_1 units. Then they may either accumulate as derivatives of H_4PtG or be diverted into other metabolic pathways and the outcome would be a lowering of methionine formation due to the corresponding fall in conversion of C_1 units into N^5 -methyl H_4PtG_3 .

(3) Enzyme-directed competition between folates for C_1 units. The distribution of C_1 units as N^5 -methyl derivatives of H_4PtG and H_4PtG_3 also depends on the relative affinities of serine hydroxymethyltransferase and N^5N^{10} -methylene tetrahydrofolate reductase for the respective folates. If the affinity for the monoglutamates is higher than for the triglutamates, then, in the steady state, competition for enzyme sites would severely limit the conversion of C_1 units into N^5 -methyl H_4PtG_3 and the formation of methionine would again be restricted. The accumulation of N^5 -methyl H_4PtG may also limit the conversion of C_1 units into methionine methyl groups by favouring the reverse reaction of enzymes normally engaged in methyl group synthesis. In all cases the extent of inhibition would depend upon the relative concentrations of the competing folates.

The purpose of the present work was to examine this inhibition in an attempt to determine its site and mode of action.

METHODS AND MATERIALS

Growth of organisms and preparation of enzyme-containing extracts. Methods of maintenance, growth and subsequent preparation of ultrasonic extracts of *Escherichia coli* have been described by Guest *et al.* (1960) and Guest *et al.* (1964c). The principal organism used in this work was *E. coli* PA15, an auxotroph requiring serine or glycine. Other auxotrophic strains lacking enzymes required for methionine biosynthesis were also used: *E. coli* 3/62, a methionine-requiring strain lacking N^5N^{10} -methylene tetrahydrofolate reductase, and *E. coli* 121/176, which requires methionine or cobalamin for growth and lacks the N^5 -methyl H_4PtG_3 -homocysteine methyltransferase. The basal medium was supplemented with glycine (10 mM) for strain PA15, DL-

methionine (0.04 mM) for strains 121/176 and 3/62; alternatively, cobalamin (18.5 μ M) was the supplement for strain 121/176. Cobalamin was sometimes added to the basal medium of the other strains to invoke the formation of holocobalaminmethyltransferase.

For the purpose of this work *E. coli* PA15 may be considered a wild-type organism because it possesses all the enzymes necessary for the formation of methionine from serine and homocysteine. A mixture of extracts of *E. coli* 3/62 and 121/176 (grown with methionine as the only supplement) has the same properties as the extract of *E. coli* PA15; H_4PtG inhibits the mixed system in the same way and this inhibition is overcome by the addition of cobalamin or by growth of either contributory strain in the presence of cobalamin (Foster *et al.* 1964). Thus extracts of *E. coli* 3/62 can be used for studying serine hydroxymethyltransferase and N^5 -methyl H_4PtG_3 methyltransferase individually, and *E. coli* 121/176 provides material for studying serine hydroxymethyltransferase and N^5N^{10} -methylene tetrahydrofolate reductase. Dialysed ultrasonic extracts were treated immediately before use with Dowex 1 (X8; Cl⁻ form; 100–200 mesh) resin by the method of Kisliuk & Woods (1960); protein concentration was measured by the method of Layne (1957).

Test for overall synthesis of methionine. The basal reaction mixture contained (μ moles in a final volume of 1 ml.): potassium phosphate buffer, pH 7.8, 125; DL-homocysteine, 7.5; L-serine, 5; fructose 1,6-diphosphate, 6; glucose, 6; $MgSO_4$, 5; ATP, 5; NAD, 0.5; FAD, 0.2; pyridoxal phosphate, 0.25; ultrasonic extract equivalent to 5 mg. of protein was added. When N^5N^{10} -methylene tetrahydrofolate was the substrate, pyridoxal phosphate was omitted and the homocysteine concentration was halved. In some experiments cobalamin (1.5 μ moles) was added to activate the cobalaminmethyltransferase system. Glucose, fructose 1,6-diphosphate, NAD and FAD are substrates for generating reduced FAD, the ultimate reducing substrate for methylene tetrahydrofolate reductase; *S*-adenosylmethionine required as a cofactor in the cobalaminmethyltransferase system is now known to be formed from ATP and trace contaminating quantities of methionine present in commercial specimens of homocysteine. Reaction mixtures were incubated at 37° for 1.0–2.5 hr. in an atmosphere of H_2 . After heating at 100° for 3 min. the reaction mixtures were centrifuged and the supernatant fluid was used for the estimation of methionine. The results are expressed in terms of $m\mu$ moles of L-methionine formed/mg. of protein;

Scheme 1. Diagrammatic representation of the pathways of methionine methyl group formation in *E. coli*.

the value obtained (about 5 mμmoles/mg. of protein) for a control containing all components of the reaction mixture but heated at zero time has been deducted.

Assay of serine hydroxymethyltransferase. This enzyme was assayed isotopically by estimating the formation of glycine from serine by the method of Bull (1963). The reaction mixture contained (in 0.5 ml.): L-[G-¹⁴C]serine (2.5 μmoles, 0.5 μCi); tetrahydrofolate (1 μmole); potassium phosphate buffer, pH 7.8 (50 μmoles); pyridoxal phosphate (0.5 μmole); ultrasonic extract equivalent to 5 mg. of protein. The reaction mixtures were incubated for 10 min. at 37° in air and stopped by adding 0.5 ml. of 2,4-dinitrophenylhydrazine reagent (0.15%, w/v, in 2N-HCl), which also formed a derivative of any pyruvate present. Samples (0.1 ml.) were chromatographed on Whatman no. 4 paper by descending technique with butan-1-ol-acetone-water-diethylamine (10:10:5:2, by vol.). Glycine was located by radioautography with Blue Brand X-ray film (Kodak Ltd.) and on test strips containing authentic glycine by using a ninhydrin spray. The radioactive glycine was measured directly with a mica end-window Geiger-Müller tube (General Electric Co., type 2B2); counting at an efficiency of 3.0% was for sufficient time to ensure a standard error of less than 5%. The results are expressed as glycine formed (counts/min./mg. of protein).

Assay of N⁵N¹⁰-methylenetetrahydrofolate reductase. This enzyme was assayed with ultrasonic extracts of *E. coli* 121/176 (methionine-grown); the conversion of N⁵N¹⁰-methylenetetrahydrofolate into N⁵-methyltetrahydrofolate was measured by the subsequent formation of methionine in a two-stage reaction with an intermediate heat treatment (Guest *et al.* 1964a). Ultrasonic extracts of *E. coli* 3/62 grown with methionine in either the absence or the presence of cobalamin were used to assay N⁵-methylH₄PtG₃ or N⁵-methylH₄PtG respectively.

The basal reaction mixture for N⁵-methyltetrahydrofolate formation contained (μmoles in 1.5 ml. final volume): N⁵N¹⁰-methylenetetrahydrofolate, 2; phosphate buffer, pH 7.8, 100; FAD, 0.1; NAD, 0.1; ethanol, 40; alcohol dehydrogenase, 150 μg.; ultrasonic extract of *E. coli* 121/176, 5 mg. of protein; when serine hydroxymethyltransferase and the reductase were studied together L-serine (5) and pyridoxal phosphate (0.25) were also added. The reaction mixture was incubated at 37° for 1 hr. under H₂ and the reductase was inactivated by heating (65°; 6 min.). The methylfolates were assayed immediately by conversion into methionine. For N⁵-methylH₄PtG₃: DL-homocysteine, 8; MgSO₄, 5; potassium phosphate buffer, pH 7.8, 100; 5 mg. of protein of an extract of *E. coli* 3/62 (supplying N⁵-methylH₄PtG₃-homocysteine methyltransferase) was added (final vol. 2.5 ml.); incubation was for 1.5 hr. at 37° under H₂. For N⁵-methylH₄PtG₃ plus N⁵-methylH₄PtG: ATP, 5; glucose, 6; fructose 1,6-diphosphate, 6; DL-homocysteine, 8; potassium phosphate buffer, pH 7.8, 100; 5 mg. of protein of an ultrasonic extract of *E. coli* 3/62, which had been grown with methionine plus cobalamin (supplying N⁵-methylH₄PtG₃ methyltransferase and cobalaminmethyltransferase), was added; incubation was in a final volume of 2.5 ml. for 1.5 hr. at 37° under H₂. In both cases the reaction was stopped by heating at 100° for 3 min. and methionine was assayed microbiologically (see below).

Assay of N⁵-methylH₄PtG₃-homocysteine methyltransferase. The methylation of homocysteine by N⁵-methyl-

H₄PtG₃ was measured either by microbiological assay of methionine or isotopically by using N⁵[¹⁴C]-methylH₄PtG₃- and radioautographic techniques. In the former, the reaction mixture contained (μmoles in 1 ml.): N⁵-methyl-H₄PtG₃, 1; potassium phosphate buffer, pH 7.8, 100; DL-homocysteine, 12; MgSO₄, 5; 1 mg. of protein from *E. coli* 3/62. Incubation was for 15 min. at 37° in air, and the reaction was stopped by heating at 100° for 3 min.; methionine was assayed microbiologically. In the isotopic experiments the conditions were similar except that only 0.15 mg. of protein was added (0.4 mg. for *E. coli* PA15) and N⁵-[¹⁴C]-methylH₄PtG₃ (0.15 μmole; 75 μmCi). Deproteinized samples (0.1 ml.) were chromatographed on Whatman no. 4 paper by the descending technique with butan-1-ol-acetic acid-water (4:1:5, by vol.). Methionine was located, after development, by radioautography or by addition of carrier methionine and the use of a ninhydrin spray. Isotopic procedures were as described above and the results are expressed as L-methionine formed in mμmoles or counts/min.

Assay of cobalaminmethyltransferase. Cobalamin-dependent methylation of homocysteine was assessed in a reaction mixture containing (μmoles in 1 ml.): ATP, 5; FAD, 0.1; NAD, 1; glucose, 6; fructose 1,6-diphosphate, 6; MgSO₄, 5; DL-homocysteine, 8; potassium phosphate buffer, pH 7.8, 100; 5 mg. of protein from *E. coli* 3/62 grown in the presence of cobalamin and methionine (to supply cobalaminmethyltransferase but not N⁵N¹⁰-methylenetetrahydrofolate reductase). The source of C₁ units and the folate added are reported for each experiment. Incubation was for 1.5 hr. under H₂ at 37°; the reaction was stopped by heating at 100° for 3 min. and methionine assayed microbiologically.

Estimation of methionine. The microbiological assay of methionine with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 was according to Gibson & Woods (1960); calcium leucovorin (N⁵-formylH₄PtG) was added to the basal medium (final concn. 10 μM), so that the growth response of the organism was not affected by the folic acid analogues introduced into the growth medium from reaction mixtures. In certain experiments the intense yellow colour of high concentrations of folate caused artificially high turbidity readings and to overcome this all the tube cultures in an assay were centrifuged, the supernatant fluid was carefully poured off and the bacteria were resuspended in water before measuring the turbidity.

Chemicals. Pteric acid (Pt), pteroyltriglutamate (PtG₃), aminopterin (4-aminoPtG), amethopterin (N¹⁰-methyl-4-aminoPtG), leucovorin (N⁵-formylH₄PtG) and N¹⁰-methylPtG were kindly supplied by Dr E. L. R. Stokstad and Dr H. P. Broquist (Lederle Laboratories). PtG was obtained from British Drug Houses Ltd., Poole, Dorset. N¹⁰-FormylPtG was prepared by dissolving PtG (100 mg.) in formic acid (21 M; 4 ml.) and incubated at 25° for 48 hr. in the dark. The crude product was recovered by freeze-drying.

Pteric acid was purified according to Waller *et al.* (1948) and all the other folates were purified by column chromatography on Whatman Standard Grade cellulose powder by using ammonium acetate (0.1 M, pH 7.0) saturated with 3-methylbutan-1-ol as the solvent. Folates were recovered by freeze-drying. PtG₃ (100 mg.) was further purified on a column (6 cm. × 4.5 cm.²) of triethylaminoethylcellulose (OH⁻ form) by eluting with ammonium acetate (0.8 M, pH 6.1, 200 ml.) to remove impurities and then with 1.0 M-buffer to elute PtG₃.

PtG was reduced to H_2PtG by the method of Futterman (1957) and to H_4PtG by catalytic hydrogenation in acetic acid with platinum oxide (Adams catalyst) according to O'Dell, Vandenberg, Bloom & Pfiffner (1947); both products were stored as solids at 0° under H_2 . Dihydroaminopterin ($4\text{-amino}H_2PtG$) was prepared manometrically by catalytic hydrogenation with platinum oxide in $0.15N\text{-NaOH}$, the reaction was stopped at the dihydro stage and the products were neutralized and stored under H_2 at -14° . All the tetrahydro derivatives were ($\pm$)-5,6,7,8-tetrahydrofolates; except for H_4PtG they were prepared in 20mm-sodium phosphate buffer (pH 7.0) by catalytic hydrogenation (Jones *et al.* 1961). N^5N^{10} -Methylenetetrahydrofolates were prepared by mixing the corresponding tetrahydrofolate with a 25% molar excess of formaldehyde at pH 7.0–7.8 for 5–10 min. under N_2 ; formaldehyde solutions were standardized by the method of Yoe & Reid (1941).

The N^5 -methyl derivatives of H_4Pt , H_4PtG , H_4PtG_3 and 4-amino H_4PtG were prepared by the reduction of N^5N^{10} -methylene derivatives with $NaBH_4$ as described by Sakami & Ukestins (1961) and purified by chromatography on triethylaminoethylcellulose (Guest *et al.* 1964a).

Cyanocobalamin was obtained from British Drug Houses Ltd.; it was 88% pure by spectrophotometric assay. ATP, NAD and yeast alcohol dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany), DL-homocysteine (Fluka A.-G., Buchs S.G., Switzerland), pyridoxal

phosphate (Roche Products Ltd., Welwyn Garden City, Herts.) and FAD (Sigma Chemical Co., St Louis, Mo., U.S.A.) were also commercial specimens. L-[G- ^{14}C]Serine and [^{14}C]paraformaldehyde were purchased from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Inhibition of the overall formation of methionine from serine and homocysteine. With ultrasonic extracts of *E. coli* PA15, serine as the C_1 donor and H_4PtG_3 ($40\mu M$) as the folate coenzyme, the only compounds which resembled H_4PtG in its ability to inhibit methionine formation were H_2PtG , the N^5N^{10} -methylene-, N^5 -methyl- and N^{10} -formyl-derivatives of H_4PtG and to a lesser extent N^5 -formyl H_4PtG , N^{10} -methyl H_4PtG , H_4Pt and N^5N^{10} -methylene H_4Pt (Fig. 1). No inhibition was found with unreduced folates at concentrations up to 2mm (Guest, 1960). Furthermore, H_4PtG progressively lost both its inhibitory and C_1 cofactor activities when decomposed in air and a number of pteridines (possible degradation products of H_4PtG , e.g. xanthopterin) were not antagonists (Guest, 1960; Kisliuk, 1961). Consequently, the presence

Fig. 1. Effect of reduced folates on the overall formation of methionine by ultrasonic extracts of *E. coli* PA15: (a) organisms grown in the absence of cobalamin (H_4PtG_3 , $0.04\mu M$ was present throughout as a cofactor); (b) organisms grown in the presence of cobalamin (no H_4PtG_3 present). Incubation was for 1.5 hr. Added folates were the unsubstituted ($\bullet$), N^5N^{10} -methylene- ($\circ$), N^5 -methyl- ($\blacktriangle$), N^{10} -methyl- ($\triangle$), N^5 -formyl- ($\blacksquare$) and N^{10} -formyl- ($\square$) substituted derivatives of H_4Pt (.....), H_4PtG (—) and H_2PtG (----).

of a reduced pteridine ring appears to be an essential characteristic of inhibitors. The glutamate residue was also necessary for full antagonism because H_4Pt and N^5N^{10} -methylene H_4Pt were only weakly active.

A comparison of the inhibitory activity of the derivatives of H_4PtG (Fig. 1a) with their ability to serve as folate coenzymes in the presence of cobalaminmethyltransferase (Fig. 1b) revealed a striking parallel between the two properties. Dihydropteroylglutamate was the most potent inhibitor and the best cofactor; however, it has been shown that conversion into H_4PtG is a prerequisite for the expression of these characteristics because neither was exhibited in the presence of 4-amino PtG at concentrations which inhibited dihydrofolate reductase (Guest & Woods, 1962b). The absence of an asymmetric centre at C-6 probably accounts for H_2PtG being twice as potent as H_4PtG . Table 1 compares the combined C_1 donor and cofactor activities for homocysteine methylation (in the presence and the absence of cobalamin) of a number of the folates used in the present work. The efficiency with which the monoglutamates bearing C_1 residues served as combined C_1 donors and cofactors for methionine formation (Table 1) was also directly related to their capacity for inhibition (Fig. 1a). Thus N^5N^{10} -methylene-, N^5 -methyl- and N^{10} -formyl- H_4PtG were all ready sources of the methionine methyl group and they exhibited a far greater inhibitory potential than N^5 -formyl H_4PtG , which was a poor C_1 donor; N^{10} -methyl H_4PtG , which was inactive as a C_1 donor, was also virtually ineffective as an inhibitor. Thus it would appear that the relative ease with which these compounds are converted into H_4PtG or into an intermediate in the cobalamin-dependent

pathway of homocysteine methylation governs their ability to inhibit the cobalamin-independent mechanism. However, these observations on the overall reaction do not reveal the actual site or mechanism of the inhibition nor do they indicate which derivative is the ultimate inhibitory agent.

Search for the site of H_4PtG inhibition

Direct attempts to locate the site of inhibitory action of H_4PtG and its derivatives required the use of higher concentrations of H_4PtG_3 . Accordingly, the effect of H_4PtG on formation of methionine was tested with substrate concentrations of H_4PtG_3 , N^5N^{10} -methylene H_4PtG_3 and N^5 -methyl- H_4PtG_3 (Fig. 2). Clearly, H_4PtG inhibited the reaction with serine and H_4PtG_3 but not with N^5 -methyl H_4PtG_3 as the C_1 donor and cofactor. With the N^5N^{10} -methylene derivative the results were unequivocal because it was a relatively poor source of the methionine methyl group (Table 1). Identical results were obtained when H_4PtG was replaced by N^5N^{10} -methylene H_4PtG and N^5 -methyl H_4PtG . These exploratory experiments indicate that the site of inhibition is at a stage in the formation of N^5 -methyl H_4PtG_3 because the inhibitors had apparently no effect on the trans-methylation reaction. The effects of H_4PtG and its derivatives were subsequently tested at the individual steps in the methylation of homocysteine.

N^5 -Methyl H_4PtG_3 -homocysteine methyltransferase. The most obvious site for specific enzyme inhibition is the N^5 -methyl H_4PtG_3 methyltransferase because of its unique position in the cobalamin-independent pathway. Ultrasonic extracts of the mutant *E. coli* 3/62 (grown with methionine as the only supplement) were used as a source of

Table 1. *Relative activity of tetrahydrofolate derivatives as cofactors and C_1 donors for methionine synthesis*

Tetrahydrofolates (1μ mole) were added to the basal reaction mixture for overall methionine synthesis with ultrasonic extracts of *E. coli* PA15 (5mg. of protein). Serine (5μ moles) and pyridoxal phosphate were omitted when the added folate contained a C_1 residue. Cobalamin was $1.5m\mu$ moles when present and incubation was for 2hr. The results are expressed as a percentage of the synthesis obtained with serine as C_1 donor and H_4PtG and cobalamin as cofactors.

Source of C_1 units	Relative C_1 donor plus cofactor activities (%)							
	Cobalamin.....Absent		Present		Absent		Present	
	Tetrahydrofolate derivatives of..... H_4Pt		H_4Pt	H_4PtG	H_4PtG	H_4PtG_3	H_4PtG_3	4-Amino- H_4PtG
Serine	0	2	1	100	100	100	0	0
N^5N^{10} -Methylene-	0	1	3	75	18	25	0	0
N^5 -Methyl-	0	2	2	120	120	120	0	0
N^{10} -Methyl-	—	—	0	2	—	—	0	0
N^5 -Formyl-	—	—	0	4	2	5	—	—
N^{10} -Formyl-	—	—	1	36	8	12	—	—

Fig. 2. Effect of H₄PtG on the utilization of different C₁ donors for methionine formation by ultrasonic extracts of *E. coli* PA15; incubation was for 2 hr. in a final volume of 1 ml. Sources of C₁ units and tetrahydrofolates (1 μmole) were: ●, serine (5 μmoles) plus H₄PtG₃; ○, N⁵-N¹⁰-methyleneH₄PtG₃; ▲, N⁵-methylH₄PtG₃.

this enzyme because they lack methylenetetrahydrofolate reductase and cobalaminmethyltransferase. Of the methylated folates examined here, only N⁵-methylH₄PtG₃ served as a substrate for this enzyme (Table 2). When the same compounds were tested in the cobalamin-dependent system N⁵-methylH₄PtG was the only other compound which was active (Table 2). With the standard assay for N⁵-methylH₄PtG₃-homocysteine methyltransferase no inhibition could be detected with H₄PtG or its derivatives at concentrations sixfold that of N⁵-methylH₄PtG₃. To overcome the problem of high colour corrections in the microbiological assay of methionine when high concentrations of folate were used, an isotopic method was developed. Then concentrations of several compounds in 20-fold excess of the substrate were found to have virtually no effect on this reaction (Table 3). Similar observations were made with extracts of *E. coli* PA15 as the source of the methyltransferase, indicating that simultaneous presence of the methylenetetrahydrofolate reductase had no effect. It was therefore concluded that the site of inhibition is at a stage before transmethylation.

N⁵-N¹⁰-Methylenetetrahydrofolate reductase. This enzyme was best studied in an extract of *E. coli* 121/176 which lacks N⁵-methylH₄PtG₃ methyltransferase and, after growth with methionine as supplement, also lacks cobalaminmethyltrans-

Table 2. *Methyltetrahydrofolates as substrates for N⁵-methylH₄PtG₃ methyltransferase and cobalaminmethyltransferase*

The basal reaction mixture was that used for assaying cobalaminmethyltransferase (see the Methods and Materials section) except that (I) N⁵-methylH₄PtG₃ methyltransferase (from methionine-grown *E. coli* 3/62) or (II) the cobalaminmethyltransferase system (from cobalamin-grown *E. coli* 121/176) supplied the enzyme protein (5 mg.). Incubation was for 2 hr.

Source of the methyl group (1 μmole)	L-Methionine formed (mμmoles/mg. of protein)	
	I (cobalamin-independent)	II (cobalamin-dependent)
N ⁵ -MethylH ₄ Pt	0	2
N ⁵ -MethylH ₄ PtG	0	96
N ⁵ -MethylH ₄ PtG ₃	70	70
N ¹⁰ -MethylH ₄ PtG	0	0
N ⁵ -Methyl-4-aminoH ₄ PtG	0	0
N ¹⁰ -Methyl-4-aminoH ₄ PtG	0	0

Table 3. *Effect of inhibitory analogues on the activity of N⁵-methylH₄PtG₃-homocysteine methyltransferase*

The basal reaction mixture (see the Methods and Materials section) contained N⁵[¹⁴C]-methylH₄PtG₃ (0.15 μmole; 75 mμC) and ultrasonic extract of *E. coli* 3/62 (0.15 mg. of protein). Incubation was for 15 min. and the methionine formed was assayed by incorporation of radioactivity.

Folate added (μmoles)*	10 ⁻³ × Incorporation of radioactivity into methionine (counts/min./mg. of protein/hr.)		
	0.3	1.0	3.0
H ₄ PtG	69	66	75
N ⁵ -N ¹⁰ -MethyleneH ₄ PtG	62	68	75
N ⁵ -MethylH ₄ PtG	75	79	75
H ₄ Pt	70	—	71
N ⁵ -MethylH ₄ Pt	72	—	69
4-AminoH ₄ PtG	56	64	66
N ⁵ -Methyl-4-amino-H ₄ PtG	58	73	70

* With no added folate the incorporation was 66 × 10³ counts/min./mg. of protein/hr.

ferase. Consequently, this extract can accumulate methylated tetrahydrofolates, which in turn can be assayed by the same methods used for studying the methyltransferase (for the triglutamate) and for studying the cobalamin-dependent system (for mono- and tri-glutamates together); the difference yields a value for the accumulated N⁵-methyl-H₄PtG. Unfortunately, this assay was not very satisfactory for accurate kinetic studies with low concentrations of substrate. With N⁵-N¹⁰-methyleneH₄PtG₃ as the substrate, the presence of

H₄PtG lowered the amount of N⁵-methylH₄PtG₃ formed and a corresponding increase in N⁵-methylH₄PtG production was observed (Table 4). N⁵-MethylH₄PtG itself had no effect at the concentration tested, suggesting that reversal of the reductase, by an increase in concentration of N⁵-methylH₄PtG, is not important as a mechanism for inhibition. In the presence of a fivefold excess of formaldehyde, identical results were obtained, indicating that spontaneous competition for C₁ units is not the basis of the inhibition. Serine hydroxymethyltransferase and methylenetetrahydrofolate reductase were examined in combination in similar experiments with serine as the C₁ donor and H₄PtG had precisely the same effect on the formation of N⁵-methylH₄PtG₃. Consequently, a competition between the folates for reduction at the active centre of methylenetetrahydrofolate reductase could explain the mechanism of inhibition by H₄PtG. The oxidation of N⁵-methyltetrahydrofolates by this enzyme was studied by aerobic incubation in the presence of 2,6-dichlorophenol-indophenol; the disappearance of N⁵-methyl-H₄PtG₃ was lowered by H₄PtG, as well as by N⁵-methylH₄PtG.

The methods used for studying methylenetetrahydrofolate reductase were also applied to determining the relative amounts of methylated tetrahydrofolates in the products of typical reaction mixtures used for studying the inhibition of H₄PtG of the overall conversion of serine plus homocysteine into methionine (Table 5). It can be seen that the degree of inhibition and the amount of N⁵-methyl-H₄PtG formed are directly related to the propor-

tions of H₄PtG₃ and H₄PtG in the original reaction mixture. N⁵-MethylH₄PtG₃ was not detected in any of the test incubations by assaying with the specific methyltransferase and subtracting the value for preformed methionine.

Serine hydroxymethyltransferase. Of the folates studied here only H₄PtG and H₄PtG₃ served as C₁ acceptors for the conversion of serine into glycine

Fig. 3. Folic acid derivatives and serine hydroxymethyltransferase. The enzyme was assayed as described in the Methods and Materials section with ultrasonic extracts of *E. coli* 3/62 (5 mg. of protein). Incubation was for 10 min. with: □, H₄Pt; ●, H₄PtG₃; ●, H₄PtG; ○, 4-aminoH₄PtG.

Table 4. Interaction of folate derivatives for the formation of methyltetrahydrofolates by the action of N⁵N¹⁰-methylenetetrahydrofolate reductase

Substrate (μmoles)	Folate added (μmoles)	Methylated folate formed (mμmoles)	
		N ⁵ -Methyl-H ₄ PtG ₃	N ⁵ -Methyl-H ₄ PtG
N ⁵ N ¹⁰ .	None	104	0
Methylene-H ₄ PtG ₃ (2)	H ₄ PtG (1)	96	25
	H ₄ PtG (2)	83	72
	H ₄ PtG (3)	75	95
	H ₄ PtG (8)	57	300
	N ⁵ -MethylH ₄ PtG (3)	106	—
	4-AminoH ₄ PtG (3)	62	—
N ⁵ N ¹⁰ .	N ⁵ -Methyl-4-amino-H ₄ PtG (2.5)	70	—
	None	—	216
	4-AminoH ₄ PtG (3)	—	50
Methylene-H ₄ PtG (2)	N ⁵ -Methyl-4-amino-H ₄ PtG (2.5)	—	64

Table 5. Accumulation of N⁵-methylH₄PtG in a reaction mixture inhibited by H₄PtG

Reactions were carried out in triplicate by using the basal mixture for the overall formation of methionine with ultrasonic extracts of *E. coli* PA15 (5 mg. of protein) and the tetrahydrofolates as given below. The incubation was stopped after 1 hr. by heating at 65° for 6 min. The products were then assayed for methionine, methionine plus N⁵-methylH₄PtG (with the specific homocysteine-methyltransferase) and methionine, N⁵-methylH₄PtG₃ plus N⁵-methylH₄PtG (with the cobalaminmethyltransferase system) and the amounts of each product determined by difference.

Tetrahydrofolate added (μmoles)		L-Methionine formed (mμmoles)	N ⁵ -Methyl-H ₄ PtG accumulated (mμmoles)
H ₄ PtG ₃	H ₄ PtG		
0.1	—	290	0
0.1	2.0	40	96
0.2	—	295	0
0.2	2.0	60	48
0.4	—	284	0
0.4	2.0	105	28
—	2.0	0	114

Table 6. *Effect of folate derivatives on the activity of serine hydroxymethyltransferase*

The enzyme was tested as described in the Methods and Materials section; *E. coli* 3/62 supplied the enzyme (5 mg. of protein) and H₄PtG (1 μmole) was the cofactor. Incubation time, 10 min.

Folate added (μmoles)	Glycine formed (counts/min.)
None	500
H ₄ Pt (3)	660
N ⁵ -MethylH ₄ Pt (2.5)	510
N ⁵ -MethylH ₄ PtG (4)	490
H ₄ PtG ₃ (1)	880
4-AminoH ₄ PtG (3)	620
N ⁵ -Methyl-4-aminoH ₄ PtG (2.5)	560

by serine hydroxymethyltransferase (Fig. 3); they were almost equally efficient. Compounds bearing N⁵ or N¹⁰ substituents were inactive. The enzyme was not inhibited by any of the folates tested when either H₄PtG (Table 6) or H₄PtG₃ was the C₁ acceptor. Moreover, the reaction was accelerated by several compounds which were otherwise inactive, possibly by providing C₁ acceptor for the C₁ units liberated by the folate cofactor; the formation of glycine was in fact inhibited by added formaldehyde. The rate of reaction was enhanced by two active folates but no attempts were made to determine the relative proportions of the two methylene derivatives when both H₄PtG and H₄PtG₃ were substrates because separately they appeared to be equivalent and, further, equilibration through free formaldehyde would be expected to distribute the C₁ units according to the relative concentrations and formaldehyde-binding capacities of the folates.

Formaldehyde-binding capacities of tetrahydrofolates

If the active forms of folic acid had lower capacities for binding formaldehyde than the inhibitory compounds, the antagonism could be explained in terms of a chemical competition for C₁ units at the methylenetetrahydrofolate level. Several observations favoured this view. First, N⁵N¹⁰-methyleneH₄PtG₃ was a poor source of the methionine methyl group compared with the corresponding monoglutamate (Table 1). Secondly, the most potent inhibitors were unsubstituted tetrahydrofolates or derivatives readily converted into free tetrahydrofolates, and of the folates these form the stablest compounds with formaldehyde (Blakley, 1959). The dissociation constants of several methylene derivatives were measured spectrophotometrically in potassium phosphate buffer

Table 7. *Binding of formaldehyde by H₄PtG₃ and H₄PtG*

[¹⁴C]Formaldehyde (0.25 μmole; 0.5 μc) was added to H₄PtG (0.5 μmole) and H₄PtG (see below) in potassium phosphate buffer (0.04 M; pH 7.0) containing ascorbic acid (2 mg./ml.). Samples were subjected to electrophoresis (40 min.; 100 v/cm. of Whatman no. 4 paper) in ammonium acetate (0.1 M; pH 6.1) containing ascorbic acid (2 mg./ml.). Formaldehyde was immediately fixed by a dimedone spray and the derivatives were detected by radioautography.

Folate present (μmoles)		10 ⁻³ × Formaldehyde bound to folate (counts/min.)	
H ₄ PtG ₃	H ₄ PtG	H ₄ PtG ₃	H ₄ PtG
0.5	—	27.0	0
0.5	1.0	11.3	24.5
0.5	4.0	5.2	41.5
0.5	10.0	2.5	55.0

(0.1 M, pH 7.5) with 2,3-dimercaptopropan-1-ol as the antioxidant according to Blakley (1960). H₄PtG and H₄PtG₃ gave the same value (0.05 mm) and H₄Pt, an extremely poor inhibitor, had a similar dissociation constant (0.07 mm); 4-amino-H₄PtG gave a value 0.36 mm. Obviously, different capacities for binding formaldehyde cannot account for the inhibitory interaction between the folate derivatives in methionine biosynthesis. These values represent much higher affinities for formaldehyde than those quoted by Blakley for H₂PtG (10 mm) or N⁵- and N¹⁰-substituted derivatives (400 and 14 mm respectively). In another experiment [¹⁴C]formaldehyde was mixed with twice its molar equivalent of H₄PtG₃ and increasing concentrations of H₄PtG were added. After 15 min. samples were subjected to electrophoresis to separate the mono- and tri-glutamate derivatives. The formaldehyde distributed itself according to the relative concentrations of the two folates and confirmed that their binding capacities are similar (Table 7). The same results were obtained if reactants were incubated under N₂ for 2 hr. before electrophoresis. Thus if equilibration through formaldehyde occurred during the enzymic formation of methionine the distribution of C₁ units would be directly proportional to the relative concentrations of the respective folates.

Studies with analogues of folic acid

Effects on the overall formation of methionine from serine and homocysteine. The observations made with derivatives of H₄PtG were extended to include derivatives of the folate analogues aminopterins (4-aminoPtG) and amethopterins (N¹⁰-

Fig. 4. Effect of derivatives of 4-aminoH₄PtG and other reduced folates on the formation of methionine by ultrasonic extracts of *E. coli* PA15 (grown without added cobalamin) in a final volume of 1ml. with: (a) H₄PtG₃ (0.05 μmole), incubation time 1.5 hr.; (b) H₄PtG (0.5 μmole) and cobalamin (1.5 mμmoles), incubation time 2 hr. Added folates were: 4-aminoH₄PtG, ●; N⁵N¹⁰-methylene-4-aminoH₄PtG, ○; N⁵-methyl-4-aminoH₄PtG, ◐; N¹⁰-methyl-4-aminoH₄PtG, ◑; N¹⁰-methylH₄PtG, △; H₄Pt, ● - - - ●.

methyl-4-aminoPtG). Like the inhibitors examined above, the unreduced parent compounds were without effect on the methylation of homocysteine. However, 4-aminoH₄PtG and N⁵N¹⁰-methylene-4-aminoH₄PtG were potent inhibitors; the N⁵- and N¹⁰-methyl derivatives also inhibited but to a less degree (Fig. 4a). By contrast with the derivatives of H₄PtG the inhibitory effects of these compounds were not overcome by the addition of cobalamin or the presence of cobalaminmethyltransferase, and no circumstances were found in which they could serve as cofactors or C₁ donors for homocysteine methylation (Table 1). Moreover, these compounds also inhibited the cobalamin-dependent mechanism of methionine synthesis with H₄PtG as the folic acid cofactor (Fig. 4b). This was first noted by Kisliuk (1960) with 4-aminoH₄PtG and N¹⁰-methyl-4-aminoH₄PtG. Fig. 4(b) also illustrates that H₄Pt and N¹⁰-methyl-H₄PtG, which were very poor antagonists of H₄PtG₃ and had no C₁ cofactor activity in the cobalamin-dependent reaction (Fig. 1), were also very poor antagonists of H₄PtG in this latter system.

A detailed investigation by the method of Lineweaver & Burk (1934) showed that, in all the cases tested, the inhibition was due to competition between the folate derivatives, e.g. the effect of

Table 8. *Kinetic analysis of the inhibition*

The basal reaction mixture for the overall formation of methionine from serine and homocysteine was used with ultrasonic extracts of *E. coli* PA15 and either (I) H₄PtG₃ as cofactor or (II) H₄PtG and cobalamin (1.5 mμmoles) were added. Kinetic data were analysed by the method of Lineweaver & Burk (1934) as in Fig. 5 and the constants are tabulated below (μM).

Cofactors	Cofactor and inhibitor constants for the overall formation of methionine (μM)	
	I (H ₄ PtG ₃)	II (H ₄ PtG + cobalamin)
K _m for tetrahydrofolate	7	70
K _i for inhibitors		
H ₄ PtG	15	—
4-AminoH ₄ PtG	2.2	1.9
N ¹⁰ -Methyl-4-amino-H ₄ PtG	25	27

4-aminoH₄PtG on the utilization of H₄PtG₃ and H₄PtG (Fig. 5). The inhibition constants, K_i, derived from these experiments are shown in Table 8. It can be seen that 4-aminoH₄PtG is the most potent inhibitor and equally active against H₄PtG

Fig. 5. Effect of 4-aminoH₄PtG on methionine formation by ultrasonic extracts of *E. coli* PA15 with (a) H₄PtG₃ as cofactor and (b) H₄PtG as cofactor in the presence of cobalamin (1.5 μM), final volume 1 ml. [S], Concentration of H₄PtG₃ or H₄PtG (mM); *V*, rate of L-methionine formation (*V*₀, μmoles/5 mg. of protein/1.5 hr.; *V*_i, μmoles/5 mg. of protein/2 hr.). Concentrations of 4-aminoH₄PtG (μM): ●, nil; ○, 5; ◐, 12.5; ◑, 15; ◒, 25; ◓, 30.

and H₄PtG₃. N¹⁰-Methyl-4-aminoH₄PtG had about one-tenth the capacity for inhibition, being less effective than H₄PtG in the cobalamin-independent system.

The competitive nature of the inhibition of tetrahydrofolates by 4-aminoH₄PtG and N¹⁰-methyl-4-aminoH₄PtG contrasted with the non-competitive inhibition of methionine formation by the unreduced 4-amino analogue when dihydrofolates were the cofactors (Guest & Woods, 1962b). Consequently, the relative effects of several derivatives of 4-aminoPtG were compared, with H₂PtG and H₄PtG as the cofactors for methionine synthesis (Fig. 6). Tested against H₂PtG, the tetrahydro derivatives of 4-aminoPtG had only 1–2% of the potency of the unreduced compounds and they exhibited the same order of inhibitory activity when tested against H₄PtG. The greater antagonism exerted by the tetrahydro derivatives against H₂PtG may have been due to trace contamination with 4-aminoPtG or 4-aminoH₂PtG. By a similar argument, the presence of traces of 4-aminoH₄PtG in the 4-aminoH₂PtG may explain why 4-aminoH₂PtG has a greater antagonism than 4-aminoPtG against H₄PtG but was less active against H₂PtG. Thus it was concluded that 4-aminoPtG and its

dihydro derivative inhibited dihydrofolate reductase whereas 4-aminoPtG inhibited a stage in the methylation of homocysteine.

Effects on specific steps in methionine synthesis. Because 4-aminoH₄PtG and its derivatives resembled H₄PtG as competitive inhibitors, it was tempting to speculate that they have a similar mechanism of action. However, these compounds exerted the same effect on H₄PtG as on H₄PtG₃ so either they must inhibit cobalaminmethyltransferase and N⁵N¹⁰-methyleneH₄PtG₃ methyltransferase in the same manner or, more probably, they must act at a site common to both mechanisms of homocysteine methylation, i.e. at stages leading to the formation of N⁵-methyltetrahydrofolates. In attempts to locate the site of inhibition of 4-aminoH₄PtG, experiments paralleling those reported above for H₄PtG were performed. Experiments with substrate concentrations of tetrahydrofolates clearly showed that 4-aminoH₄PtG inhibited the use of serine and N⁵N¹⁰-methyleneH₄PtG but not N⁵-methylH₄PtG as C₁ donor (Fig. 7); similar results were obtained with the N⁵N¹⁰-methylene- and N⁵- and N¹⁰-methyl derivatives of 4-aminoH₄PtG. With derivatives of H₄PtG₃ as substrates the same effects were observed except the results

Fig. 6. Effect of derivatives of 4-aminoPtG on the formation of methionine by ultrasonic extracts of *E. coli* PA15. The basal reaction mixture contained H_2PtG (---) or H_4PtG (—) as cofactors ($0.5 \mu\text{mole}$) and cobalamin ($1.5 \mu\text{moles}$) in a final volume of 1 ml. Incubation was for 2.5 hr., during which time $0.86 \mu\text{mole}$ of L-methionine was formed (■). Added folate analogues were: ○, 4-aminoPtG; ◐, 4-amino- H_2PtG ; ●, 4-amino- H_4PtG ; ▲, N^5 -methyl-4-amino- H_4PtG .

with N^5N^{10} -methylene- H_4PtG_3 were not as clear because this is a poor C_1 donor (see Table 1 and Fig. 2). Further evidence suggesting that inhibition occurs before transmethylation was provided by direct tests on the triglutamate-specific methyltransferase (Table 3) and the cobalaminmethyltransferase; no inhibition was detected, nor were the methyl groups of N^5 - or N^{10} -methyl-4-amino- H_4PtG sources of the methionine methyl group (Table 2).

A direct inhibitory effect of 4-amino- H_4PtG and N^5 -methyl-4-amino- H_4PtG was found by examining the formation of N^5 -methyl- H_4PtG_3 and N^5 -methyl- H_4PtG from the corresponding methylene derivatives, the reaction catalysed by methylenetetrahydrofolate reductase (Table 4). These compounds also inhibited the reverse reaction but no attempt was made to determine whether they could serve as substrates. Derivatives of 4-amino-PtG were neither cofactors (Fig. 4) nor inhibitors (Table 6) for serine hydroxymethyltransferase.

DISCUSSION

Inhibition by H_4PtG and its derivatives. In addition to inhibition by H_4PtG , cobalamin-independent methylation of homocysteine was

Fig. 7. Effect of 4-amino- H_4PtG on the utilization of different C_1 donors for the formation of methionine by ultrasonic extracts of *E. coli* PA15 with H_4PtG or derivative ($1 \mu\text{mole}$) plus cobalamin ($1.5 \mu\text{moles}$) in a final volume of 1 ml. Incubation was for 2 hr. C_1 donors were: serine ($5 \mu\text{moles}$), ●; N^5N^{10} -methylene- H_4PtG , ◐; N^5 -methyl- H_4PtG , ▲.

inhibited by a number of monoglutamate derivatives, particularly those most readily converted into H_4PtG by enzymic processes known to occur in ultrasonic extracts of *E. coli*. Foliates more oxidized than H_2PtG , derivatives of H_4Pt (lacking a glutamate residue) and compounds having metabolically unreactive C_1 substituents, were either inert or only weak antagonists. The capacity for inhibition was in fact directly related to a compound's ability to serve as a cofactor or substrate in cobalamin-dependent homocysteine methylation. Direct studies with serine, N^5N^{10} -methylene- and N^5 -methyl-tetrahydrofolate as substrates indicated that the site of inhibition was at some stage in the formation of methyl groups rather than in transmethylation. Although N^5 -methyl- H_4PtG was found to accumulate in reaction mixtures inhibited by H_4PtG , specific inhibition of N^5 -methyl- H_4PtG_3 -homocysteine methyltransferase could not be demonstrated. Furthermore, no inhibitory compounds were found to affect the transmethylation catalysed by this enzyme. The possibility of a feed-back effect of N^5 -methyl- H_4PtG or serine hydroxymethyltransferase or N^5N^{10} -methylenetetrahydrofolate reductase was also excluded as a mechanism for inhibition. There was no correlation between the ability of a compound to inhibit methionine synthesis and its capacity for binding formaldehyde, because the N^5N^{10} -methylene derivatives of H_4PtG (an inhibitor) and H_4Pt

(inert) had similar dissociation constants and these in turn were equal to the value for N^5N^{10} -methylene- H_4PtG_3 . Thus it was impossible to explain the inhibition in terms of a chemical competition for C_1 units based on differences in affinity for C_1 units between the folates.

Since the mono- and tri-glutamates are both substrates for the enzymes concerned with methyl group synthesis the possible mechanisms of inhibition must be based upon differences in the affinity of the enzyme for these substrates or simply upon differences in the relative concentrations of the folates. As for serine hydroxymethyltransferase, the two substrates were equally efficient so it would be expected that the distribution of C_1 units at the hydroxymethyl level would be proportional to the relative concentrations of H_4PtG and H_4PtG_3 . Preferential reduction of N^5N^{10} -methylene- H_4PtG by the methylenetetrahydrofolate reductase was suspected because this was a better source of C_1 units than the corresponding triglutamate for the overall formation of methionine. However, direct measurement of the methylated products formed from mixtures of the two substrates (Table 4) indicated that the reduction of the two substrates was directly related to their initial concentrations. Consequently, the inhibition of cobalamin-independent methionine synthesis by H_4PtG is best explained as a competition between the two folic acid cofactors, based on concentration, for C_1 units and their reduction at serine hydroxymethyltransferase and methylenetetrahydrofolate reductase. When the relative activities of the enzymes in a typical extract of *E. coli* PA15 are considered, the following reaction rates are found to correspond to an overall rate of methionine production (0.1 μ mole/mg. of protein/hr.) with H_4PtG_3 as cofactor; serine hydroxymethyltransferase (2.5 μ moles/mg./hr.); N^5N^{10} -methylenetetrahydrofolate reductase (0.1–0.2 μ mole/mg./hr., approximately); N^5 -methyl- H_4PtG_3 methyltransferase (0.3 μ mole/mg./hr.). The rate-limiting step is clearly the reduction of C_1 units. Furthermore, the rapid liberation of C_1 units from serine would be expected to permit equilibration of the C_1 units according to the concentration of the respective folates. Consequently, the critical site of competition between the folates for determining the rate of N^5 -methyl- H_4PtG_3 formation would seem to be the reduction catalysed by methylenetetrahydrofolate reductase. The steady-state kinetics of a multi-enzyme system with two competing substrates is exceedingly complex and at this stage a thorough kinetic analysis of the problem is not possible. However, the most plausible explanation for the observed inhibition is a direct competition between the N^5N^{10} -methylenetetrahydrofolates for methylenetetrahydrofolate reductase, based on the relative

concentrations of the two substrates and not, as was once supposed, a direct effect of the monoglutamates on N^5 -methyl- H_4PtG -homocysteine methyltransferase. It is not known whether this competition has any physiological significance; it could form the basis of a mechanism of metabolic regulation.

Inhibition by 4-amino- H_4PtG and its derivatives. A study of inhibition by 4-amino- H_4PtG indicated that the inhibitory effect of H_4PtG was probably a special case of a general inhibitory phenomenon. The unique property of H_4PtG and its derivatives was their ability to function as cofactors or substrates in the alternative or cobalamin-dependent mechanism of methionine synthesis. Derivatives of 4-amino- H_4PtG were inactive in this respect and, consequently, they inhibited both mechanisms of methionine synthesis in what appeared to be a similar and a competitive manner. For this reason it was predicted that their site of action is common to both pathways and therefore at some stage in the synthesis of the methyl group rather than in transmethylation. Direct confirmation of this emerged from a study of the enzymes concerned; the inhibitors had no effect on N^5 -methyl- H_4PtG_3 methyltransferase or on the cobalaminmethyltransferase system. Serine hydroxymethyltransferase was likewise unaffected but N^5N^{10} -methylenetetrahydrofolate reductase was inhibited by 4-amino- H_4PtG and by N^5 -methyl-4-amino- H_4PtG with the N^5N^{10} -methylene derivative of either H_4PtG or H_4PtG_3 as substrate. Whether or not N^5N^{10} -methylene-4-amino- H_4PtG was reduced by this enzyme was not determined. However, it is clear that tetrahydroaminopterin inhibits methionine synthesis by a competitive interaction with the substrates of N^5N^{10} -methylenetetrahydrofolate reductase and this limits the formation of N^5 -methyltetrahydrofolates. In this respect, it is similar to H_4PtG , which exerts its effect by limiting the formation of N^5 -methyl- H_4PtG_3 . The high potency of 4-amino- H_4PtG is probably a reflexion of the effect of the 4-amino group on the affinity between the inhibitor and the reductase. This is supported by the fact that the N^5 - and N^{10} -methyl derivatives of 4-amino- H_4PtG inhibit in circumstances in which the corresponding derivatives of H_4PtG were found to be inactive. The effect of 4-amino- H_4PtG could certainly not be related to its capacity for binding formaldehyde because the dissociation constant of its methylene derivative was higher than those of the derivatives of H_4PtG and H_4PtG_3 .

The competitive inhibition by 4-amino- H_4PtG of tetrahydrofolate-catalysed methionine synthesis contrasted with the non-competitive inhibition exerted by 4-amino- PtG when dihydrofolate was the cofactor (Guest & Woods, 1962b). Dihydro-

aminopterin and 4-aminoH₄PtG inhibited the utilization of H₂PtG at concentrations one-hundredth and one three-hundredth of the concentrations of 4-aminoH₄PtG needed to inhibit H₄PtG and H₂PtG respectively. It was therefore concluded that 4-aminoPtG and 4-aminoH₂PtG specifically inhibited dihydrofolate reductase and that 4-aminoH₄PtG inhibited a step in methyltetrahydrofolate formation. The greater activity of 4-aminoH₄PtG found with H₂PtG as the coenzyme source was probably due to contamination by the more oxidized forms. Similar conclusions about the action of aminopterin and reduced derivatives have been reached by Zakrezewski, Hakala & Nichol (1962).

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