- Epstein, S. S. & Weiss, J. B. (1960). *Biochem. J.* **75**, 247.
- Foster, M. A., Jones, K. M. & Woods, D. D. (1961*a*). *Biochem. J.* **80**, 519.
- Foster, M. A., Rowbury, R. J. & Woods, D. D. (1963). *J. gen. Microbiol.* **31**, xix.
- Foster, M. A., Tejerina, G. & Woods, D. D. (1961*b*). *Biochem. J.* **81**, 1*p*.
- Gibson, F. & Woods, D. D. (1960). *Biochem. J.* **74**, 160.
- Gorini, L. & Maas, W. K. (1958). In *The Chemical Basis of Development*, p. 469. Ed. by McElroy, W. D. & Glass, B. Baltimore: The John Hopkins Press.
- Guest, J. R. (1960). *Biochem. J.* **76**, 405.
- Guest, J. R., Foster, M. A. & Woods, D. D. (1964*a*). *Biochem. J.* **92**, 488.
- Guest, J. R., Friedman, S., Foster, M. A., Tejerina, G. & Woods, D. D. (1964*b*). *Biochem. J.* **92**, 497.
- 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.
- Guest, J. R. & Woods, D. D. (1962*a*). 2 *Europäisches Symposium über Vitamin B₁₂ und Intrinsic Factor*, p. 686. Ed. by Heinrich, H. C. Stuttgart: Ferdinand Enke Verlag.
- Guest, J. R. & Woods, D. D. (1962*b*). *Biochem. J.* **82**, 26.
- Hatch, F. T., Cathou, R. E. & Larrabee, A. R. (1960). *Fed. Proc.* **19**, 417.
- Hatch, F. T., Larrabee, A. R., Cathou, R. E. & Buchanan, J. M. (1961). *J. biol. Chem.* **236**, 1095.
- Hatch, F. T., Takeyama, S. & Buchanan, J. M. (1959). *Fed. Proc.* **18**, 243.
- Jones, K. M., Guest, J. R. & Woods, D. D. (1961). *Biochem. J.* **79**, 566.
- Kisliuk, R. L. (1960). *Biochim. biophys. Acta*, **40**, 531.
- Kisliuk, R. L. (1961). *J. biol. Chem.* **236**, 817.
- 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.
- Nakao, A. & Greenberg, D. M. (1958). *J. biol. Chem.* **230**, 603.
- Stevens, A. & Sakami, W. (1959). *J. biol. Chem.* **234**, 2063.
- Szulmajster, J. & Woods, D. D. (1960). *Biochem. J.* **75**, 3.
- Takeyama, S. & Buchanan, J. M. (1960). *Fed. Proc.* **19**, 417.
- Wilmanns, W., Rücker, B. & Jaenicke, L. (1960). *Hoppe-Seyl. Z.* **322**, 283.

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

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

The general background of the present work was fully reviewed in the preceding paper (Foster, Tejerina, Guest & Woods, 1964), in which was also presented experimental evidence that there are alternative pathways for the conversion of homocysteine into methionine by strains of *Escherichia coli*. These differ in the mechanism by which the C₁ unit is transferred (with concomitant reduction) from N⁵N¹⁰-methylene-tetrahydrofolate to homocysteine (Scheme 1 in Foster *et al.* 1964). In one case only N⁵N¹⁰-methylene-H₄PtG₃ could be used as substrate; it was converted by an enzyme *A* into

an unidentified intermediate (X₃) that reacted with homocysteine in the presence of an enzyme *B*; cobalamin derivatives were not required at either step. The alternative mechanism was dependent on a cobamide-containing enzyme, clearly distinguishable from enzyme *B*. In this case the N⁵N¹⁰-methylene derivative of either H₄PtG or H₄PtG₃ was an effective substrate and they were converted by enzyme *A* into intermediates X₁ and X₃ respectively; both X₁ and X₃ yielded methionine when incubated with homocysteine and an enzymic extract possessing the cobamide-containing enzyme but not enzyme *B*.

The object of the present work was to identify the intermediates X₁ and X₃ and to study in more detail the mechanism of their formation by enzyme *A*. *E. coli* 121/176 (an auxotroph giving a growth response with either methionine or cobalamin) was used as a specific source of this enzyme since it lacks enzyme *B* and possesses no cobamide-containing enzyme when grown with methionine (Table 1).

The technique used for the identification of X₁ (and consequently of X₃) was aided by the publica-

* Abbreviations for members of the folic acid group, based on PtG for pteroylglutamate: H₄PtG, tetrahydropteroylmonoglutamate; H₄PtG₃, tetrahydropteroyltriglutamate; H₂PtG, dihydropteroylglutamate; derivatives of these compounds are indicated as, e.g., N⁵N¹⁰-methylene-H₄PtG, N⁵-methyl-H₄PtG₃.

tion, while this work was in progress, of brief communications implicating a methyl derivative of tetrahydrofolate, probably N^5 -methyl- H_4 PtG, as an intermediate in methionine synthesis, first in pig liver (Sakami & Ukstins, 1961) and then in cell-free extracts of strains of *E. coli* (Larrabee, Rosenthal, Cathou & Buchanan, 1961). Meanwhile it had also been shown (Keresztesy & Donaldson, 1961; Donaldson & Keresztesy, 1961) to be identical with a form of folic acid (prefolic A) present in horse liver.

MATERIALS AND METHODS

Preparation of enzyme-containing extracts. The two auxotrophic strains of *E. coli* (121/176 and 3/62) used in this work were as described by Foster *et al.* (1964); their content of relevant enzymes is summarized in Table 1. The methods used for their maintenance and growth, and for the preparation of ultrasonic extracts from the harvested organisms were as described by Guest, Helleiner, Cross & Woods (1960). The medium used for the bulk growth of both strains was normally supplemented with DL-methionine (0.4 mM); under these conditions neither strain possessed the cobamide-containing enzyme. When extracts in which the cobamide-containing enzyme was present were required the supplements used were cobalamin alone for strain 121/176, and cobalamin plus methionine for strain 3/62. The cobalamin was separately sterilized and added (final concn. 18.5 μ M) to the previously autoclaved bulk medium. The crude dialysed extracts could be stored at -14° for up to 10 weeks without significant decrease of activity with respect to either enzyme A or enzyme B.

Several methods were used, either singly or in combination, to remove substances of the coenzyme type from the enzyme preparations immediately before they were used. Treatment with Dowex 1 resin (Cl⁻ form; X8; 100–200 mesh) followed the procedure of Kisliuk & Woods (1960). The technique of Kisliuk (1960) was used for gel-filtration on columns of Sephadex G-50 (see below). The method proved satisfactory with small volumes (5–8 ml.) of enzyme preparations containing no more than 15 mg. of protein/ml. Ground and acid-washed Nuchar C (West Virginia Pulp and Paper Co., New York, U.S.A.) was used for treatment with charcoal. An aqueous suspension (2.5 mg./ml.) was shaken for 16 hr. and the wetted particles were collected by centrifuging (at 25 000g for 5 min.). The enzyme preparation (10–20 mg. of protein/ml.) was stirred at 0° for 20 min. with an amount of the prepared charcoal (per mg. of protein) equivalent to 0.2–0.5 mg. of the original dry charcoal. The enzymes were recovered by centrifuging (at 25 000g for 5 min.) to remove charcoal.

Purified enzyme A was obtained by the procedure of Foster *et al.* (1964); the material used (fraction III; 80-fold purification) contained 25 μ g. of protein/ml. It was further treated to remove cofactors by gel-filtration as described above.

Enzymic formation and assay of X_1 and X_3 . Conditions affecting the formation of these intermediates by preparations of enzyme A were studied in a basal reaction mixture (M_1) containing (in final vol. 1 ml.): N^5N^{10} -methylene- H_4 PtG (3 μ moles) or N^5N^{10} -methylene- H_4 PtG₃ (2 μ moles); potassium phosphate buffer, pH 7.8 (125 μ moles); enzyme preparation equivalent to 5 mg. of protein (or 2.5 μ g. plus 2.5 mg. of albumin when the purified enzyme was used). The NADH₂-generating system, when added, consisted of NAD (1 μ mole), ethanol (40 μ moles) and crystallized alcohol dehydrogenase (150 μ g.; C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany). Other supplements, when present, were ATP (5 μ moles), MgSO₄ (5 μ moles), FAD (0.1 μ mole) and FADH₂ (1.5 μ moles). The last-named substance was generated *in situ* after the remainder of the reaction mixture containing FAD (1.5 μ moles) had been placed under anaerobic conditions in a Thunberg tube at 0° ; solid Na₂S₂O₄ (12 μ moles) was then added from a side arm. The completed reaction mixtures were incubated for 1.5 hr. at 37° under an atmosphere of H₂; the enzymic reaction was terminated by heating at 65° for 7 min.

The basal reaction mixture (M_2) used for the enzymic assay of X_1 and X_3 contained (in final vol. 2 ml.): DL-homocysteine, 7.5 μ moles; glucose, 20 μ moles; ATP, 10 μ moles; MgSO₄, 10 μ moles; NAD, 1 μ mole; FAD, 0.1 μ mole; potassium phosphate buffer, pH 7.8, 250 μ moles ATP, MgSO₄, NAD and FAD were omitted, and the amount of potassium phosphate was decreased to 125 μ moles, when the assay was on unpurified samples derived directly from reaction mixtures containing these substances. The reaction mixture was completed by the addition of samples judged to contain 0.05–2.5 μ moles of X_1 or X_3 and an ultrasonic extract of *E. coli* 3/62 (treated with Dowex 1) equivalent to 5 mg. of protein. The extract was derived from organisms grown with and without cobalamin for the assay of X_1 and X_3 respectively. The complete reaction mixture was incubated in an atmosphere of H₂ for 1.5 hr. at 37° . After heating (at 100° for 3 min.) the supernatant fluid after centrifuging was used for the assay of methionine by the microbiological method of Gibson & Woods (1960). The amount of methionine formed was directly proportional to the amount of X_1 or X_3 added, although the reaction with homocysteine was not necessarily quantitative. The amount of X_1 and X_3 present is therefore expressed in terms of the L-methionine (μ moles) formed in the assay. The value obtained in controls with heated enzyme varied from 5 to 10 μ moles; this has been deducted from all the results given.

Column chromatography. Triethylaminoethylcellulose (TEAE-cellulose) (Serva Entwicklungslabor, Heidelberg, Germany) was used for the purification, isolation and identification of the various folates encountered in the present work. Fine particles were removed by repeated decantation of an aqueous suspension and the final slurry was packed into columns (24 cm. $\times$ 4.6 cm.²) under a pressure of N₂ of 10 lb./in.². The columns were washed (or regenerated) by passing through double the column volume of 0.5N-NaOH followed by water until the effluent was neutral; the final flow rate was about 50 ml./hr. with a head of 30–40 cm. of fluid. Samples containing up to 100 μ moles of folate were

Table 1. *Distribution of enzymes in auxotrophic strains of Escherichia coli*

Strain of <i>E. coli</i>	Supplement for growth	Enzyme			Cobamide-containing enzyme
		A	B		
121/176	Methionine	+	—	—	
	Cobalamin	+	—	+	
3/62	Methionine	—	+	—	
	Methionine + cobalamin	—	+	+	

chromatographed; the developing agent was ammonium acetate buffer, pH 6.1, standardized by estimating the NH_4^+ ion concentration (Russell, 1944), and 2-mercaptoethanol (40 mM) was added when reduced folates were present. The loaded columns were first washed with ammonium acetate buffer (200 ml.) of the chosen initial concentration; the folates were then eluted by increasing the salt concentration by means of a linear-gradient device (Bock & Ling, 1954) with a mixing vessel of capacity 450 ml. Fractions (10 ml.) of the effluent were collected successively with an automatic fraction collector; direct estimation of NH_4^+ ion concentration served to check whether the desired gradient was being obtained. After locating fractions containing folates by the methods described below, the fractions associated with a given peak were pooled, desalted by freeze-drying and stored under H_2 in the solid state or as solutions in 50 mM-potassium phosphate buffer, pH 7.8.

Initial purification of the PtG and PtG₃ used for the preparation of tetrahydro derivatives was achieved on columns of Whatman cellulose powder (standard grade). A slurry in 100 mM-ammonium acetate buffer, pH 7, saturated with isopentanol was packed into a column (45 cm. $\times$ 9 cm.²) and washed with the suspending fluid under a pressure of N_2 of 15 lb./in.². Solutions containing 100–200 mg. of folate were applied and eluted with the same solvent at a flow rate of 50 ml./hr.

Sephadex G-50 dextran gel (Pharmacia, Uppsala, Sweden) was used both to deproteinize reaction products containing unstable folates and to free enzyme preparations from cofactors. Fine particles were removed by repeated decantation of aqueous suspensions. The column (15 cm. $\times$ 1.7 cm.²) was poured as a slurry, allowed to settle and, before the sample was applied, washed with twice the column volume of the developing fluid (either 50 mM-potassium phosphate buffer, pH 7.8, or 40 mM-2-mercaptoethanol).

All columns were developed in a cold room (0–4°) and protected from light.

Spectrophotometry. The extinction ($E_{1\text{ cm.}}$) of fractions obtained after column chromatography was measured at 280 m μ with a Unicam SP. 500 spectrophotometer, with the eluting fluid in the blank cuvette. Absorption spectra of selected fractions and pooled material were obtained with a Cary recording spectrophotometer model 14 M-50.

Isotopic technique. The radioactivity of fractions derived from the chromatography of [¹⁴C]folates was measured with a thin mica end-window Geiger-Müller tube. Samples (0.1 ml.) were plated directly on disks of lens tissue (no. 105; J. B. Green Ltd., Maidstone, Kent) fixed to aluminium planchets (3 cm.²) and counted for a sufficient time to ensure a standard error of less than 5% with a counting efficiency of 3.5% (Francis, Mulligan & Wormall, 1954); the background count was deducted.

Tetrahydrofolates. H₄PtG was prepared from PtG (folic acid; British Drug Houses Ltd., Poole, Dorset) by catalytic hydrogenation by the method of Kisliuk & Woods (1960). Smaller quantities of purer material were obtained by first removing a fluorescent impurity from PtG by column chromatography on cellulose powder; after removing ammonium acetate by freeze-drying the sample was hydrogenated on a small scale in 20 mM-potassium phosphate buffer, pH 7, by the method described by Jones, Guest & Woods (1961) for H₄PtG₃.

Two samples of PtG₃ were used during this work for the

preparation (on a small scale) of solutions of H₄PtG₃ (Jones *et al.* 1961). Both were synthetic products and were kindly provided by Dr E. L. R. Stokstad. Sample I had been stored in this Laboratory as a solid for several years and was considerably purer than sample II which was a solution (10 mg./ml.) containing preservative. Chromatography on cellulose powder removed most of the fluorescent impurities from both samples, but not the brown colour of sample II. H₄PtG₃ prepared from treated sample I contained only small amounts of ultraviolet-absorbing impurities as judged by examination of the fractions obtained after chromatography on TEAE-cellulose (gradient 0.35–0.7 M- NH_4^+ ion). The product from treated sample II, however, contained, besides H₄PtG₃ (eluted at 0.62 M- NH_4^+ ion), several other compounds, one of which (eluted at 0.44 M- NH_4^+ ion) accounted for about 30% of the total ultraviolet-absorbing material eluted. These less pure samples of H₄PtG₃ were often used directly for the chemical preparation of derivatives (see below); since high yields were obtained the derivatives themselves could be finally purified by chromatography on TEAE-cellulose.

Purer H₄PtG₃ was, however, required for the study of the enzymic formation of its derivatives. Chromatography of PtG₃ (both samples) on TEAE-cellulose yielded a pale-yellow product which was eluted at 1.4–1.6 M- NH_4^+ ion and which was freed from ammonium acetate by freeze-drying. H₄PtG₃ prepared from this contained insignificant amounts of other pteridine derivatives when sample I was used, and with sample II only a small proportion of the original impurity remained.

The N⁵N¹⁰-methylene derivatives of H₄PtG and H₄PtG₃ were prepared in solution as required by incubating the folate for 5–10 min. under N_2 with a 25% molar excess of formaldehyde in potassium phosphate buffer, pH 7.8.

Other chemicals. A solution of [¹⁴C]formaldehyde (25 mCi; 2 $\mu\text{Ci}/\mu\text{mole}$) was obtained by heating an aqueous suspension of [¹⁴C]paraformaldehyde (0.6 $\mu\text{Ci}/\mu\text{g.}$; The Radiochemical Centre, Amersham, Bucks.) in a sealed tube at 120° for 24 hr.

Crystalline cyanocobalamin was obtained from British Drug Houses Ltd., and ATP, NAD, FAD and FMN from the Sigma Chemical Co., St Louis, Mo., U.S.A. DL-Homocysteine came from either Mann Research Laboratories Inc., New York, U.S.A., or A.-G. Fluka, Buchs SG, Switzerland.

Assay of folates. The microbiological methods of Jones *et al.* (1961) were used except that freshly prepared ascorbic acid (adjusted to pH 6.5) was added to the basal medium at a final concentration of 1 mg./ml. The standards used were PtG and PtG₃ for the assay of mono- and tri-glutamate forms of folic acid respectively by *Lactobacillus casei*, PtG for *Streptococcus faecalis* R and N⁵-formyl-H₄PtG for *Pediococcus cerevisiae*. At least three determinations were made for each experimental sample with each organism.

RESULTS

Formation of the intermediates

Cofactor requirements for the formation of X₁ and X₃ from the N⁵N¹⁰-methylene derivatives of H₄PtG and H₄PtG₃ respectively were first investigated with a preparation of enzyme A (derived from *E. coli* strain 121/176 grown with methionine;

Table 1) from which low-molecular-weight components had been removed by successive treatments with Dowex 1 resin, charcoal and Sephadex G-50; it had not, however, been fractionated with respect to protein. The amount of X_1 and X_3 produced was judged by the amount of methionine formed on further incubation with a source of the cobamide-containing enzyme or enzyme *B* respectively. To keep conditions as similar as possible these two enzymes were both derived from the same organism (*E. coli* strain 3/62) grown under the appropriate conditions (Table 1). Both FAD and a system regenerating NADH_2 were required for the maximum formation of X_1 and X_3 , but these could be replaced by substrate concentrations of chemically reduced FAD (Table 2).

With purified enzyme *A*, the system producing NADH_2 was no longer effective even in the presence of FAD; substrate quantities of FADH_2 were now essential (Table 3). A requirement for a bivalent metal ion could not be detected even after further treatment of the purified enzyme by shaking it gently at 0° for 2 hr. with Chelex-100 (Dowex A 1) chelating resin (K^+ form; 50–100 mesh).

Isolation of the intermediates

The preparation of X_1 and X_3 was carried out on a scale 5–10 times as great as that used in the detailed study of their formation. Typically, the substrate (N^5N^{10} -[^{14}C]methylene-tetrahydrofolate) was prepared *in situ* by adding [^{14}C]formaldehyde (20 μmoles ; about 0.3 $\mu\text{C}/\mu\text{mole}$) to the remainder of the reaction mixture which contained (in final vol. 5 ml.): H_4PtG or H_4PtG_3 (16 μmoles), FAD (8 μmoles), potassium phosphate buffer, pH 7.8 (400 μmoles), and a preparation of enzyme *A* equivalent to 16 mg. of protein and obtained by treatment of a dialysed extract of *E. coli* strain 121/176 with Dowex 1 resin and charcoal. The mixture was divided equally between two Thunberg tubes each fitted with two side arms that contained respectively freshly prepared alkaline pyrogallol and solid sodium dithionite (30 μmoles); the latter was tipped in after flushing the tubes with hydrogen. After incubating for 1.5 hr. at 37° the enzymic reaction was terminated by cooling the tubes to 0° and 2-mercaptoethanol (300 μmoles) was added to each tube. Protein was removed from the pooled products with Sephadex G-50 and the low-molecular-weight components were eluted from the column with 40 mM-2-mercaptoethanol; the yellow flavin served to locate the material. After chromatography on TEAE-cellulose the radioactivity and extinction at 280 $m\mu$ were measured with each fraction; complete spectra were recorded for peak fractions, both as collected and after adjustment to pH 1 and pH 11, for comparison with authentic folates.

With N^5N^{10} -methylene- H_4PtG as substrate only the product eluted at 0.3M- NH_4^+ ion (peak B in Fig. 1) had the properties of X_1 , i.e. ability to act directly as a methyl donor to homocysteine in the absence of enzyme *A*; it also contained the bulk of the radioactivity associated with any fraction containing folate (Fig. 1). Peak A was FAD, and peak C had the chromatographic properties corresponding to either the original substrate or H_4PtG ; it was presumably the latter since it had negligible radioactivity and did not act, even in the presence of

Table 2. *Enzymic formation of X_1 and X_3 : requirement for a reducing system*

The source of enzyme *A* was an extract of strain 121/176 treated to remove cofactors, but not otherwise purified. Basal reaction mixture M_1 was supplemented with the substances indicated at concentrations stated in the Materials and Methods section. The X_1 and X_3 formed were assayed enzymically as described in that section.

Substrate	Amount of product (in terms of $\mu\text{m-moles}$ of L-methionine)	
	N^5N^{10} -Methylene- H_4PtG X_1	N^5N^{10} -Methylene- H_4PtG_3 X_3
Product...		
Supplement to reaction mixture		
FAD, 'NADH ₂ '*, ATP, MgSO_4	180	—
FAD, 'NADH ₂ '*	184 (5†)	135 (7†)
FAD	18	17
'NADH ₂ '*	132	106
FADH_2	230 (5†)	—

* 'NADH₂' refers to the NADH_2 -generating system comprising NAD, ethanol and alcohol dehydrogenase.

† Values obtained with a heated preparation of enzyme *A*.

Table 3. *Formation of X_1 and X_3 by purified enzyme A: requirement for reduced flavin-adenine dinucleotide*

The conditions were as for Table 2, except that a fraction of extract of strain 121/176 was used that had been purified as to enzyme *A*.

Substrate	Amount of product (in terms of $\mu\text{m-moles}$ of L-methionine)	
	N^5N^{10} -Methylene- H_4PtG X_1	N^5N^{10} -Methylene- H_4PtG_3 X_3
Product ...		
Reducing system used		
FADH ₂		
+	113 (3†)	115 (3†)
+	131	109
—	16	5
—	12	4

* 'NADH₂' refers to the NADH_2 -generating system comprising NAD, ethanol and alcohol dehydrogenase.

† Values obtained with a heated preparation of enzyme *A*.

enzyme *A*, as a precursor of the methyl group of methionine. It is probable that the [^{14}C]methylene group of residual unchanged substrate is removed during chromatography by reaction with the 2-mercaptoethanol (Blakley, 1958) of the eluting fluid, yielding H_4PtG and [^{14}C]thioacetals; the appearance of radioactivity in the early fractions (Fig. 1), where the extinction at $280\text{ m}\mu$ is due to 2-mercaptoethanol, supports this view.

Similar experiments with the triglutamate form of N^5N^{10} -tetrahydrofolate as substrate are illustrated by Fig. 2. The component of the reaction products (peak B) with the biological properties of X_3 contained the bulk of the radioactivity associated with folates; it was eluted with 0.53 M-NH_4^+ ion and was clearly separated both from X_1 and from H_4PtG_3 (peak C) derived during chromatography from unchanged substrate.

The clear-cut separations described above depend on the use of a preparation of enzyme *A* free from NAD and on the use of FADH_2 as direct hydrogen donor. If NAD and a NADH_2 -generating system are used with catalytic quantities of FAD the results are confused by the concurrent oxidation, presumably by a NAD-linked reaction, of the N^5N^{10} -methylene-tetrahydrofolates to a mixture of the corresponding N^5 - and N^{10} -formyl derivatives. The N^5 -formyl derivatives of H_4PtG and H_4PtG_3 appeared in the same fractions as X_1 and X_3 respectively, whereas the N^{10} -derivatives were eluted before FAD.

Chemical reduction of N^5N^{10} -methylene-tetrahydrofolates

Since X_1 and X_3 are formed enzymically from N^5N^{10} -tetrahydrofolates by the sole addition of a catalytic reducing system or FADH_2 , a comparison of their properties with products obtained by chemical reduction was essential. The method used was based on those of Sakami & Ukstins (1961) and Keresztesy & Donaldson (1961). A solution of N^5N^{10} -[^{14}C]methylene- H_4PtG ($25\text{--}70\text{ mM}$; $1.2\text{ }\mu\text{C}/\mu\text{mole}$) or the corresponding triglutamate derivative (8 mM ; $0.3\text{ }\mu\text{C}/\mu\text{mole}$) was prepared in 50 mM -potassium phosphate buffer, pH 7, and brought to 45° in a stream of nitrogen. Solid sodium borohydride was added in portions to a final concentration of 0.2 and 0.1 M respectively, and the incubation continued for 1.5 hr . The products were neutralized and, if not analysed immediately, freeze-dried and stored at -14° under nitrogen.

The distributions of radioactivity and extinction at $280\text{ m}\mu$ in fractions after chromatography on TEAE-cellulose are illustrated in Figs. 3 and 4 for the mono- and tri-glutamates respectively. The major radioactive product from N^5N^{10} -methylene- H_4PtG eluted at the same concentration of NH_4^+ ions (0.3 M) as did X_1 (Fig. 3), and that from N^5N^{10} -

methylene- H_4PtG_3 (Fig. 4) at the same concentration (0.53 M) as X_3 . As in the enzymic experiments, residual substrate lost the radioactive methylene group and appeared as non-radioactive H_4PtG or H_4PtG_3 . The non-radioactive ultraviolet-absorbing material that was eluted at 0.44 M-NH_4^+ ion (Fig. 4) was the unidentified major impurity present in H_4PtG_3 when prepared from the less pure sample (II) of PtG_3 .

Fig. 1. Chromatography on TEAE-cellulose of enzymic reaction products containing the intermediate X_1 . —, Extinction at $280\text{ m}\mu$; - - -, radioactivity.

Fig. 2. Chromatography on TEAE-cellulose of enzymic reaction products containing the intermediate X_3 . —, Extinction at $280\text{ m}\mu$; - - -, radioactivity.

Comparison of enzymically reduced and chemically reduced N^5N^{10} -methylenetetrahydrofolates

Physical properties. As noted above X_1 and X_3 were identical with the products of the chemical reduction of the N^5N^{10} -methylene derivatives of H_4PtG and H_4PtG_3 respectively with regard to the salt concentration at which they were eluted from TEAE-cellulose. The absorption spectra of all four

Fig. 3. Chromatography on TEAE-cellulose of the products of chemical reduction of N^5N^{10} -methylene- H_4PtG . —, Extinction at 280 $m\mu$; ----, radioactivity.

Fig. 4. Chromatography on TEAE-cellulose of the products of chemical reduction of N^5N^{10} -methylene- H_4PtG_3 . The original starting material was PtG_3 purified from sample I. —, Extinction at 280 $m\mu$; ----, radioactivity.

materials were closely similar, when measured immediately after elution from the column, at either pH 6.1 or 1.0 (Fig. 5). At pH 6.1 the extinction was maximal at 290 $m\mu$ and minimal at 245 $m\mu$. Progressive decrease in the pH resulted in a decrease in the extinction at 290 $m\mu$ and a shift of the maximum to 292 $m\mu$; below pH 4 a second maximum appeared at 268–269 $m\mu$, and at pH 1.0 there were minima at 244 and 279 $m\mu$. The maximum extinction was slightly greater at pH 7.0 than at pH 6.1; at pH 10, though unchanged in magnitude, it had shifted to 287 $m\mu$.

Activity in the methylation of homocysteine. The chemically reduced methylenetetrahydrofolates were used at twice the concentration of X_1 and X_3 on the assumption (later justified) that, being derived from racemic precursors, they would be likely to have, at most, half the activity of the natural products. The stated concentration of the test solutions of all four materials was based on spectrophotometric assay assuming an arbitrary value of 25 000 $cm^2/mole$ for the molar extinction coefficient at the absorption maximum (290 $m\mu$) at neutral pH; this value is similar to that obtained with other folates.

Both X_1 (derived from N^5N^{10} -methylene- H_4PtG) and the corresponding chemically reduced compound methylated homocysteine, but only if the cobamide-containing enzyme was present in the enzyme preparation used (Table 4). Thus preparations of *E. coli* strain 3/62 were inactive if the organism were grown without cobalamin, but

Fig. 5. Ultraviolet-absorption spectra of the products of enzymic (----) and chemical (—) reduction of N^5N^{10} -methylenetetrahydrofolates at pH 6.1 (A) and pH 1.0 (B). The spectra of the mono- and tri-glutamate forms could not be distinguished.

Table 4. Comparison of chemically reduced and enzymically reduced N^5N^{10} -methylenetetrahydrofolates as source of the methyl group of methionine

The conditions used for the study of methionine synthesis were those described in the Materials and Methods section for the assay of X_1 and X_3 , but with as enzyme source extracts of the stated strains of *E. coli* grown with or without cobalamin as indicated. Amount of substrate added (for basis of assessment see the text): chemically reduced derivatives, 1.0 μ mole; X_1 , 0.5 μ mole; X_3 , 0.4 μ mole.

Strain of <i>E. coli</i>	L-Methionine formed (μ m-moles)			
	3/62		121/176	
	Absent	Present	Absent	Present
Cobalamin in growth medium				
Substrate				
N^5N^{10} -Methylene- H_4 PtG after reduction:				
(a) Enzymically (i.e. X_1)	7	473	15	456
(b) Chemically	5	445	18	430
N^5N^{10} -Methylene- H_4 PtG ₃ after reduction:				
(a) Enzymically (i.e. X_3)	250	310	—	—
(b) Chemically	363	477	21	420

active if cobalamin had been present. A similar difference was found with preparations of strain 121/176 grown under the two conditions (Table 4) and also with ultrasonic extracts of *E. coli* strain PA 15. It is clear that enzyme *B*, which is present in strain 3/62 grown without cobalamin (Table 1), is inactive with the chemically reduced substrate as well as that (X_1) formed enzymically.

A source of enzyme *B*, however, is sufficient to catalyse the methylation of homocysteine with the triglutamate forms of the substrate (X_3 and its synthetic counterpart), and the cobamide-containing enzyme is not required in either case (Table 4). Thus extracts of strain 3/62 grown without cobalamin are effective and methionine synthesis is increased by only 20–30 % in the concurrent presence of the cobamide-containing enzyme (from organisms grown with cobalamin). Extracts of strain 121/176 (which lacks enzyme *B*) are inactive unless derived from organisms grown with cobalamin and thus possessing the cobamide-containing-enzyme system which can also use the triglutamates as substrate.

A quantitative comparison of the relative activities (under the appropriate conditions) of the natural and synthetic compounds is illustrated in Fig. 6. With both mono- and tri-glutamate forms of substrate the natural material was about twice as effective as the synthetic; in terms of moles of L-methionine formed/mole of folate added the values were 0.93 and 0.44 for the H_4 PtG derivatives and 0.38 and 0.19 for those derived from H_4 PtG₃. A similar difference was found when they were tested, not as substrates, but as catalysts of the overall conversion of serine plus homocysteine into methionine by extracts of *E. coli* PA 15; the method of Jones *et al.* (1961) was used. The apparent K_m values were 93 and 172 μ M respectively for the natural and synthetic monoglutamate derivatives, and 4.6 and 9.5 μ M for the triglutamates.

Fig. 6. Relative activity of the products of enzymic and chemical reduction of N^5N^{10} -methylenetetrahydrofolates as methyl donors for methionine synthesis. The conditions used were those described in the Materials and Methods section for the assay of X_1 and X_3 . —, H_4 PtG derivatives; ---, H_4 PtG₃ derivatives. O, Enzymic-reduction products (X_1 and X_3); ●, chemical-reduction products.

Growth-factor activity for bacteria. The reduced methylene derivatives (of both H_4 PtG and H_4 PtG₃) effectively supported the growth of *Lactobacillus casei*, but not that of the other folate-requiring organisms tested. As expected, the synthetic materials had only half the activity of those formed enzymically (Table 5). They appeared to be a little more active than PtG, but it is possible that the arbitrary value chosen for the extinction coefficient (and which determined the stated concentration)

Table 5. *Growth-factor activity for Lactobacillus casei of chemically reduced and enzymically reduced N^5N^{10} -methyltetrahydrofolates*

The results are expressed as percentages of the activity shown by an equivalent molar concn. of PtG and PtG₃ respectively for the H₄PtG and H₄PtG₃ derivatives.

	Relative growth-promoting activity
N^5N^{10} -Methylene-H ₄ PtG after reduction:	
(a) Enzymically (i.e. X ₁)	132
(b) Chemically	66
N^5N^{10} -Methylene-H ₄ PtG ₃ after reduction:	
(a) Enzymically (i.e. X ₃)	105
(b) Chemically	54

was too low. With *Pediococcus cerevisiae* (which does not respond to PtG) all four compounds had less than 0.1 % of the activity of N^5 -formyl-H₄PtG, the required growth factor. With *Streptococcus faecalis* R the synthetic materials had less than 1 % of the activity of PtG, but the enzymic products had significantly greater activity (up to 1 %); this may have been due to incomplete chromatographic separation from active compounds as a consequence of the relatively low yields obtained enzymically.

DISCUSSION

The conversion of N^5N^{10} -methylene-H₄PtG into X₁ by purified preparations of enzyme A is dependent only on the presence of substrate quantities of FADH₂. Assuming that only one isomer (with reference to the asymmetric C-6 of the tetrahydropteridine residue) is biologically active, X₁ was qualitatively and quantitatively similar to the product of chemical reduction of N^5N^{10} -methylene-H₄PtG both with regard to physicochemical properties (chromatographic behaviour and ultraviolet-absorption spectrum) and with regard to biological activity as source of the methyl group of methionine and as growth factor for *Lactobacillus casei*. It is clear therefore that the enzymic reaction producing X₁ is a simple reduction. X₁ is also similar in absorption spectrum and growth-factor activity to the prefolic A (natural and synthetic) of Keresztesy & Donaldson (1961) and to the compound implicated by Larrabee *et al.* (1961) as an intermediate of methionine synthesis in other strains of *E. coli*. It is generally agreed that these compounds, and also the synthetic material shown by Sakami & Ukstins (1961) to be a precursor of the methyl group of methionine with pig-liver preparations, are all N^5 -methyl-H₄PtG. The presence of a *N*-methyl group has been demonstrated, and the inability of the substance to combine with formaldehyde suggests

that there is substitution at the N^5 - or N^{10} -position (Larrabee *et al.* 1961; Donaldson & Keresztesy, 1961). The relative stability of the compound indicates a N^5 rather than a N^{10} derivative; N^{10} -methyl-H₄PtG has previously been shown to be inactive as a source of the methyl group of methionine in the present test systems (Guest, 1960), and this is also the case both in another *E. coli* system and in pig liver (Larrabee *et al.* 1961; Sakami & Ukstins, 1961).

Enzyme A of *E. coli* is thus a N^5N^{10} -methylene-tetrahydrofolate reductase catalysing the following reaction:

It is specific for FADH₂, though the tetrahydrofolate may be either in the mono- or tri-glutamate form. Evidence for reversibility comes from the observation (J. R. Guest, unpublished work) that N^5 -methyltetrahydrofolates disappear when incubated in air with 2,6-dichlorophenol-indophenol and unpurified enzyme. A similar enzyme in mammalian liver, so far studied only with monoglutamate derivatives, catalyses the reaction in either direction according to whether it is coupled to the reduction of menadione or to the oxidation of NADH₂ (Donaldson & Keresztesy, 1961, 1962*a*). The enzyme may therefore also be termed a N^5 -methyltetrahydrofolate dehydrogenase.

It has been shown by Donaldson & Keresztesy (1962*b*) that N^5 -methyl-H₂PtG is rapidly and non-enzymically reduced to the tetrahydro derivative by thiols such as mercaptoethanol and homocysteine. The former was present throughout the manipulations used for the isolation of X₁ and X₃, and the latter was necessarily present as substrate in the enzymic tests for methionine synthesis with X₁ and X₃ as methyl donors. If N^5 -methyl-H₂PtG were the true product of enzyme A, the reaction catalysed would be:

that is, a single-step change requiring no added reducing system; enzyme A, however, has an obligatory requirement for FADH₂ or a system which can generate it. Further, Kisliuk (1963), in experiments on methionine synthesis from serine and homocysteine by *E. coli* PA15 with tetra-deuteropteroylglutamate as cofactor, found that deuterium did not appear in methionine. It is clear therefore that N^5 -methyltetrahydrofolates are the direct products of the action of enzyme A.

The metabolic defect in *E. coli* auxotrophs of the 3/62 type (i.e. giving a growth response with

methionine but not also with cobalamin) can now be defined as their inability to synthesize N^5N^{10} -methylene-tetrahydrofolate reductase and thus to produce N^5 -methyltetrahydrofolates; eight other auxotrophs with the same growth characteristics had a similar disability. Such strains contain an enzyme (*B*) which transfers the methyl group from N^5 -methyl- H_4PtG_3 to homocysteine. On the other hand methionine auxotrophs which also grow with cobalamin (e.g. strain 121/176) contain the reductase but not enzyme *B*; they cannot use N^5 -methyl- H_4PtG_3 as methyl donor unless grown with cobalamin. These results support the hypothesis of alternative pathways elaborated by Foster *et al.* (1964) and locate the point at which the two pathways diverge as subsequent to the creation of the methyl group. A detailed study of the two mechanisms by which the methyl group is transferred to homocysteine from N^5 -methyltetrahydrofolates is the subject of the next paper (Guest, Friedman, Foster, Tejerina & Woods, 1964).

SUMMARY

1. The products formed from N^5N^{10} -methylene-tetrahydropteroyl-monoglutamate and -triglutamate respectively, by an enzyme (*A*) absent from auxotrophs of *Escherichia coli* responding to methionine only (but present in other strains), have been isolated by chromatographic methods and characterized as the corresponding N^5 -methyl derivatives. The identification was based on a comparison with chemically prepared material of their physical and biochemical properties.

2. By using purified preparations of the enzyme the other reactant was found to be, specifically, $FADH_2$.

3. N^5 -Methyltetrahydropteroyltriglutamate methylates homocysteine in the presence of an enzyme *B* which is lacking in cobalamin/methionine auxotrophs. The monoglutamate derivative does not act as substrate for this enzyme, but is effective (as is also the triglutamate) with extracts of organisms grown in the presence of cobalamin and possessing a cobamide-containing enzyme.

4. The metabolic lesion in the methionine auxotrophs examined that respond to methionine only can be definitely ascribed to lack of enzyme *A* (N^5N^{10} -methylene-tetrahydrofolate reductase).

5. The results support a previous conclusion that there are alternative enzymic mechanisms for the formation of methionine from homocysteine and show that the point of divergence is subsequent to the creation of the methyl group.

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REFERENCES

- Blakley, R. L. (1958). *Nature, Lond.*, **182**, 1719.
- Bock, R. M. & Ling, N.-S. (1954). *Analyt. Chem.* **26**, 1543.
- Donaldson, K. O. & Keresztesy, J. C. (1961). *Biochem. biophys. Res. Commun.* **5**, 289.
- Donaldson, K. O. & Keresztesy, J. C. (1962*a*). *J. biol. Chem.* **237**, 1298.
- Donaldson, K. O. & Keresztesy, J. C. (1962*b*). *J. biol. Chem.* **237**, 3815.
- Foster, M. A., Tejerina, G., Guest, J. R. & Woods, D. D. (1964). *Biochem. J.* **92**, 476.
- Francis, G. E., Mulligan, W. & Wormald, A. (1954). *Isotopic Tracers*, p. 294. London: The Athlone Press.
- Gibson, F. & Woods, D. D. (1960). *Biochem. J.* **74**, 160.
- Guest, J. R. (1960). D.Phil. Thesis: University of Oxford.
- Guest, J. R., Friedman, S., Foster, M. A., Tejerina, G. & Woods, D. D. (1964). *Biochem. J.* **92**, 497.
- Guest, J. R., Helleiner, C. W., Cross, M. J. & Woods, D. D. (1960). *Biochem. J.* **76**, 396.
- Jones, K. M., Guest, J. R. & Woods, D. D. (1961). *Biochem. J.* **79**, 566.
- Keresztesy, J. C. & Donaldson, K. O. (1961). *Biochem. biophys. Res. Commun.* **5**, 286.
- Kisliuk, R. L. (1960). *Biochim. biophys. Acta*, **40**, 531.
- Kisliuk, R. L. (1963). *J. biol. Chem.* **238**, 397.
- Kisliuk, R. L. & Woods, D. D. (1960). *Biochem. J.* **75**, 467.
- Larrabee, A. R., Rosenthal, S., Cathou, R. E. & Buchanan, J. M. (1961). *J. Amer. chem. Soc.* **83**, 4094.
- Russell, J. A. (1944). *J. biol. Chem.* **156**, 457.
- Sakami, W. & Uktins, I. (1961). *J. biol. Chem.* **236**, 4050.