means of anion-exchange HPLC as described in published work from this laboratory.[35] Nucleosides were not retained on a Whatman Partisil-10 SAX HPLC column, whereas the 5'-monophosphates of 5-FdUrd and C-5-F-2'-dUrd were retained for 7 min on a linear gradient (40 min) of $NH_4H_2PO_4$ (5 mM, pH 2.8, to 750 mM, pH 3.7) at a flow rate of 2 mL/min. The dUMP analogues were separated from other UV-absorbing components of the enzyme reaction mixture, primarily adenine nucleotides, so that quantitation of nucleotide formation was possible.

Enzymatic phosphorylation of [6-^{3}H]dUrd by enzyme preparations from L-1210 cells was examined by the kinase assay method used above,[34] and effects of the deoxyuridine analogues on such phosphorylation was determined. Enzymatic phosphorolysis of deoxyuridine, 5-FdUrd, and C-5-F-2'-dUrd in 0.05 M sodium phosphate buffer, pH 7.2, was examined by means of reverse-phase HPLC on a Waters C_{18} μBondapak column using 5 mM $NH_4H_2PO_4$, pH 2.5, as an isocratic eluant (0.4 mL/min). The retention times (min) for uracil (9), 5-FUra (9.4), dUrd (15), 5-FdUrd (16.6), and C-5-F-2'-dUrd (18) were such that the enzymatic conversion of nucleoside to base could be followed by means of a UV detector (254 nm). Deoxyuridine and 5-FdUrd were readily phosphorylyzed to Ura and to 5-FUra, respectively, but conversion of C-5-F-2'-dUrd to 5-FUra was not detectable.

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Synthesis of 5,11-Methenyltetrahydrohomofolate and Its Antifolate Activity in Vitro

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The synthesis of 5,11-methenyltetrahydrohomofolate was accomplished by treatment of tetrahydrohomofolate (H_4homofolate) with triethyl orthoformate in glacial acetic acid. This compound is a homofolate analogue of 5,10-methenyltetrahydrofolate which serves as one precursor to the 10-formyl one-carbon donor for the first transformylation in de novo purine biosynthesis, namely, the conversion of glycinamide ribonucleotide (GAR) to *N*-formylglycinamide ribonucleotide (FGAR), catalyzed by the enzyme glycinamide ribonucleotide transformylase (EC 2.1.2.2). The analogue proved to retard the rate of formation of formylglycinamide ribonucleotide apparently by inhibiting the rate of synthesis of 10-formyltetrahydrofolate, the actual cofactor for the transformylase enzyme, from 5,10-methenyltetrahydrofolate. Its inhibition of the enzyme, 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), was competitive against (+)-L-5,10-methenyltetrahydrofolate, with a K_i = 41 μM. This derivative of homofolate may be responsible for its inhibition of purine biosynthesis in Sarcoma 180 cells.

Homofolate has been found to be a potent inhibitor of *N*-formylglycinamide ribonucleotide (FGAR) biosynthesis in Sarcoma 180 cells.[1] However, maximal expression of inhibition required prior incubation of the cells with homofolate for 24 h. Under these conditions, it was found that 20–40 μM homofolate inhibited the incorporation of [2-^{14}C]glycine into FGAR by 20–90%. Coadministration of 5-formyl-H_4folate (0.01 and 0.1 mM) and homofolate (1–200 μM) resulted in competitive protection against inhibition for FGAR biosynthesis, while administration of 5-formyl-H_4folate (0.1–10 mM) subsequent to a 24-h incubation with homofolate (10 μM) resulted in only a slight reversal of inhibition of FGAR biosynthesis.[1] These results suggested that a metabolite of homofolate was the actual inhibitor and that the enzyme GAR transformylase was the target of inhibition in this cell line. Reduced homofolates, such as H_2homofolate and H_4homofolate, appeared not to be solely responsible for the observed inhibition, since a cell subline containing 300 times more dihydrofolate reductase responded similarly.[1]

A conceivable metabolite of homofolate capable of inhibiting GAR transformylase would be the homofolate analogue of (+)L-5,10-methenyl-H_4folate that might block the synthesis of 10-formyltetrahydrofolate through inhibition of the cyclohydrolase activity of the trifunctional enzyme. The latter is one of four enzymes, along with the two tetrahydrofolate-requiring transformylases and serine transhydroxymethylase, that comprise a possible complex of enzymes involved in de novo purine biosynthesis.[2] We recently have shown that the 10-formyl rather than the 5,10-methenyl species is the actual cofactor for the GAR transformylase from the chicken liver enzyme.[3] This report describes the synthesis of 5,10-methenyl-H_4homofolate and its effect on FGAR synthesis utilizing 5,10-methenyl-H_4folate as the assay substrate for the enzyme complex in vitro.

Chemistry. The synthesis of 5,11-methenyl-H_4homofolate was accomplished, in moderate yield, through reaction of H_4homofolate with triethyl orthoformate in glacial acetic acid. H_4homofolate was obtained from homofolate in two steps by reduction with dithionite to H_2homofolate,[4] followed by dihydrofolate reductase cata-

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lyzed reduction to H_4homofolate,[5] which resulted in the production of a single diastereomer.

The ^1H NMR spectrum (200 MHz, 1 N DCl) was consistent with the proposed structure, with a low-field singlet at δ 8.99 characteristic of the amidinium proton[5] and AA'BB' doublet of doublets, δ 7.53, 7.48, 7.16, and 7.11, with J_{AB} = 9.2 Hz, in accord with the para-disubstituted benzene ring of the *p*-aminobenzoyl glutamate moiety.[7] The UV spectrum (1 N HCl) was characterized by an absorption maximum at 317 nm (ϵ 23 000 M^{-1} cm^{-1}) and absorption at 290 nm (ϵ 19 700 M^{-1} cm^{-1}). The 5,10-methenyl-H_4folate, with λ_{max} 348 (ϵ 26 500 M^{-1} cm^{-1}) and 284 nm, is the only H_4folate derivative with a long-wavelength absorption maximum.[8]

Biological Evaluation. 5,11-Methenyl-H_4homofolate was investigated as an inhibitor of GAR transformylase in vitro. The analogue proved to inhibit the rate of formation of FGAR from 5,10-methenyl-H_4folate and GAR in the presence of an enzyme complex that contains folate cofactor synthesizing activities—the trifunctional protein and serine transhydroxymethylase—and the de novo purine biosynthetic transformylase activities. When used to challenge the 5,10-methenyl-H_4folate cyclohydrolase activity of the trifunctional enzyme, the analogue was competitive with respect to (+)L-5,10-methenyl-H_4folate, with K_i = 41 μM. No inhibition of the 5,10-methylene-H_4folate dehydrogenase was observed when 5,10-methylene-H_4folate and 5,11-methenyl-H_4homofolate were present at approximately equimolar concentrations. The analogue proved to be an inhibitor vs. 5,10-methenyl-H_4folate when the latter was used as an assay substrate for GAR transformylase with a K_i within experimental error of that for the observed cyclohydrolase activity of the trifunctional enzyme.

The controls indicated that, with the conditions employed, the cyclohydrolase-catalyzed hydrolysis of 5,11-methenyl-H_4homofolate was approximately 4% of the rate of hydrolysis of 5,10-methenyl-H_4folate. If all Bratton–Marshall[9] positive species were derived from GAR transformylase utilization of 11-formyl-H_4homofolate to give FGAR and H_4homofolate, the rate of utilization of the analogue was approximately 8% of that observed with 5,10-methenyl-H_4folate.

Discussion

Homofolate was found to exhibit time-dependent inhibition of FGAR biosynthesis in Sarcoma 180 cells, and this time dependence was attributed to metabolism of homofolate by these cells to afford the proximate inhibitor of GAR transferase.[1] A conceivable metabolite of homofolate with the potential for inhibiting GAR transformylase, aside from H_4homofolate itself, would be 5,11-methenyl-H_4homofolate or 11-formyl-H_4homofolate. Homofolate is known to be a substrate for mammalian dihydrofolate reductases, resulting in production of H_4-homofolate.[10,11]

Although the proposed inhibitory metabolite of homofolate has yet to be isolated and characterized, we have synthesized 5,11-methenyl-H_4homofolate, a possible candidate, and investigated its effect on several folate-requiring enzymes. Condensation of diastereomerically pure H_4homofolate with triethyl orthoformate produced a single diastereomer of the desired 5,11-methenyl-H_4homofolate in moderate yield from homofolate. The spectral data obtained for the product are consistent with the proposed structure. A comparison of these data to those obtained for 5,10-methenyl-H_4folate [^1H NMR (100 MHz, 0.5 N DCl) singlet amidinium proton resonance at δ 9.95 and AA'BB' system centered at δ 8.33 and 7.94 (J_{AB} = 9 Hz);[12] UV (1 N HCl) λ_{max} 348 nm (ϵ 26 500 M^{-1} cm^{-1}), 284][8] may reflect a deviation from planarity and concommitant reduction of conjugation between the *p*-aminobenzoyl glutamate moiety and the pyrimidine ring, upon progression from the five-membered imidazolium ring of 5,10-methenyl-H_4folate to the six-membered ring of 5,11-methenyl-H_4homofolate. Quaternization of N-8 is unlikely due to the lower basicity of N-8 relative to N-5[13] with the resultant formation of a seven-membered vs. a six-membered ring.

We have investigated the behavior of this newly synthesized folate analogue with two activities of the trifunctional enzyme, namely, 5,10-methenyl-H_4folate cyclohydrolase and 5,10-methylene-H_4folate dehydrogenase. 5,11-Methenyl-H_4homofolate proved to be an excellent competitive inhibitor of 5,10-methenyl-H_4folate cyclohydrolase catalyzed hydrolysis of 5,10-methenyl-H_4folate. The K_i for the analogue is 41 μM as compared to a K_m of 20 μM for the substrate, (+)L-5,10-methenyl-H_4folate. It similarly inhibited the formation of FGAR when assayed with the 5,10-methenyl-H_4folate that served as the 10-formyl precursor. This result suggests that the homofolate analogue decreases the rate of FGAR formation by inhibiting the synthesis of the required 10-formyltetrahydrofolate cofactor. If this mechanism reflects the in vivo metabolic sequence, this homofolate derivative could inhibit FGAR biosynthesis. The refractiveness of 5,11-methenylhomofolate toward enzymatic and nonenzymatic hydrolysis to the 11-formyl species does not favor its mode of action in these experiments as arising from conversion to the formyl derivative. Indeed, if the enzyme complex utilized in this study functions by "channeling" to furnish the labile folate cofactors then the points of entry would be at the level of tetrahydrofolate or its 5,10-methenyl adduct.

In conclusion, our data indicate that 5,11-methenyl-H_4homofolate is an inhibitor of 5,10-methenyl-H_4folate cyclohydrolase in vitro. This homofolate derivative, or its poly(γ-glutamyl) conjugate, as recently shown for methotrexate[14] may be the metabolite(s) of homofolate indirectly responsible for inhibition of GAR transformylase and, consequently, de novo purine biosynthesis in Sarcoma 180 cells.

Experimental Section

Folic acid, *d,l*-L-H_4folate, α-ketoglutarate, glucose 6-phosphate, β-mercaptoethanol (β-ME), $NADP^+$, Tris, bovine liver glutamate dehydrogenase (EC 1.4.1.3), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were purchased from Sigma Chemical

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Co., St. Louis, MO. Maleic acid and triethyl orthoformate were obtained from Aldrich Chemical Co., Milwaukee, WI. DEAE-cellulose (DE-52) was from Whatman, Clifton, NJ, and phosphocellulose (Cellex-P) was from Bio-Rad Laboratory, Richmond, CA. TLC plates (Cellulose 13254 with fluorescent indicator) were purchased from Eastman, Rochester, NY. All other reagents were the highest quality commercially available. Doubly distilled, deionized water was used throughout. Dihydrofolate reductase (DHFR) (EC 1.5.1.3) was the generous gift of Dr. C. R. Matthews, The Pennsylvania State University, University Park, PA. Homofolate, NSC 79249, was provided through the courtesy of Dr. H. Wood, National Cancer Institute, Bethesda, MD, and was also synthesized according to literature procedures.[15] (+)L-5,10-Methenyl-H_4folate was prepared[16] from (−)L-H_4folate, produced by DHFR-catalyzed reduction of H_2folate,[5] which in turn was prepared by dithionite reduction of folic acid.[17] GAR was synthesized as described previously.[18]

Synthesis of 5,11-Methenyltetrahydrohomofolate. Homofolate (100 mg, NSC 79249) was purified by the method of Hahas and Friedkin,[10] and the UV spectrum was in accord with that reported.[19] H_2homofolate was generated from homofolate by dithionite reduction[12] and yielded the expected UV spectrum.[20] DHFR-catalyzed reduction of H_2homofolate[3] produced H_4-homofolate, which was purified by gradient elution from DEAE-cellulose with a linear gradient made from 0.2 M ammonium bicarbonate, pH 8.0, 0.1 M β-ME (700 mL), and 0.7 M ammonium bicarbonate, pH 8.0, 0.1 M β-ME (700 mL). The spectrum agreed with that reported,[20] and the yield of H_4-homofolate was 92 μmol (42 mg) based on 295 nm (ϵ 20 500 M^{-1} cm^{-1}).[20] The product was isolated by evaporation (0.5 mm, 50 °C) of the buffer after conversion to the more volatile triethylammonium bicarbonate by addition of triethylamine (2-fold excess). The residue was dissolved in glacial acetic acid (15 mL) and treated with triethyl orthoformate (1 mL, 6 mmol)[21] under argon in the dark at 25 °C. The progress of the reaction was monitored by following the increase in absorbance at 323 nm with time of aliquots of the reaction solution. When no further increase was observed (~40 h), the product was isolated by precipitation upon addition of diethyl ether (5 volumes) to the reaction solution. The product was collected by centrifugation, washed with ether (3 × 10 mL), and dried in vacuo (0.1 mm, 25 °C). Purification was achieved by elution from phosphocellulose (1.25 × 16.8 cm) with a linear gradient made from 0.05 N HCOOH, 0.01 M β-ME (170 mL), and 10 N HCOOH, 0.01 M β-ME (170 mL), followed by drying in vacuo (0.1 mm, 80 °C). The product was homogeneous by TLC on cellulose [R_f 0.285, *n*-BuOH–CH_3COOH–H_2O, 40:6:15 (v/v)]. UV (1 N HCl) λ_{max} 317 nm (ϵ 23 000), 290 (19 700). The ^1H NMR spectrum (200 MHz, 1 N DCl) showed a singlet at δ 8.99 (amidinium proton, 1 H) and an AA'BB' system at δ 7.53, 7.48, 7.16, and 7.11 (dd, J_{AB} = 9.2 Hz, *p*-ABG aromatic protons, 4 H). Anal. ($C_{22}H_{25}N_7O_8$, monoformate salt) C, H.

Enzyme Preparation and Kinetics. The enzyme preparation utilized in this study is the previously reported complex,[2] isolated from chick liver, which contains the folate cofactor synthesizing activities, 10-formyl-H_4folate synthetase (EC 6.3.4.3), 5,10-methylene-H_4folate dehydrogenase (EC 1.5.1.5), 5,10-methenyl-H_4folate cyclohydrolase (EC 3.5.4.9), and serine hydroxymethyltransferase (EC 2.1.2.1), and the de novo purine biosynthetic activities, GAR transformylase (EC 2.1.2.2) and AICAR transformylase (EC 2.1.2.3).

The kinetic studies were run at 25 °C, and initial velocities were used in the reciprocal plots (Lineweaver–Burk plots). The assays were performed essentially as described,[2] with the following modifications: the inhibition studies with GAR transformylase were run with (α + β) GAR at 0.5 mM, while the concentration of (+)L-5,10-methenyl-H_4folate was varied from 12.5 to 50 μM and the 5,11-methenyl-H_4homofolate concentration ranged from 0 to 0.1 mM. The 5,10-methenyl-H_4folate cyclohydrolase assays were performed with concentrations of (+)L-5,10-methenyl-H_4-folate ranging from 12.5 to 50 μM, while 5,11-methenyl-H_4-homofolate concentration was varied from 0 to 50 μM. The 5,10-methylene-H_4folate dehydrogenase was assayed with 25 μM (+)-5,10-methylene-H_4folate and 0–20 μM 5,11-methenyl-H_4-homofolate, while $NADP^+$ was maintained at 10 μM with a glutamate dehydrogenase couple.[23]

Controls for the 5,10-methenyl-H_4folate cyclohydrolase and the GAR transformylase inhibition studies were performed to establish the rate of buffer-catalyzed and cyclohydrolase-catalyzed hydrolysis of 5,11-methenyl-H_4homofolate and to assess the contribution of 5,11-methenyl-H_4homofolate (or its reaction products) to Bratton–Marshall[9] positive species. 5,11-Methenyl-H_4homofolate (0.15 mM) was dissolved in 50 mM maleate, pH 6.8, containing EDTA (1 mM). The decrease in A_{317nm} was monitored at 25 °C. Enzyme, at the same level used in the inhibition studies, was added and the ΔA_{317nm} was again monitored. Finally, GAR (0.5 mM) was added and the reaction was allowed to proceed for 15 min, the longest reaction time utilized in the inhibition studies. An aliquot (0.65 mL) of the reaction solution was quenched with 30% trichloroacetic acid (0.1 mL), treated with 1 N H_2SO_4 (0.1 mL), 0.1% sodium nitrite (0.05 mL), 0.5% ammonium sulfamate (0.05 mL), and 0.1% N^1-naphthylethylenediamine dihydrochloride in 0.01 N HCl (0.05 mL), and quantitated as described.[2]

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