Biochimica et Biophysica Acta, 422 (1976) 419—426

BBA 67703

INHIBITION OF DIHYDROFOLATE SYNTHETASE BY FOLATE, HOMOFOLATE, PTEROATE AND HOMOPTEROATE AND THEIR REDUCED FORMS

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(Received August 4th, 1975)

Summary

Dihydrofolate (H_2-folate) synthetase (EC 6 3 2 12) was isolated from *Escherichia coli* B A radiochemical assay was developed to determine the activity of H_2-folate synthetase in order to study the effects of folate metabolites and antimetabolites which would interfere with the microbiological assay method previously used The effects of folate and pteroate derivatives on the activity of this enzyme were investigated to determine if inhibition of this enzyme could constitute a site of action for these compounds as chemotherapeutic agents or a site of metabolic regulation. H_2-folate synthetase was inhibited by its product, H_2-folate, and by the antimetabolite dihydrohomopteroate, with apparent K_i values of 23 4 and 9 2 μM, respectively

Introduction

The biosynthesis of H_2-folate from H_2-pteroate and L-glutamic acid is catalyzed by H_2-folate synthetase (EC 6 3 2 12) [1,2] proceeding as follows

$$H_2\text{-pteroate} + \text{L-glutamate} + \text{ATP} \xrightarrow{H_2\text{-folate synthetase}} H_2\text{-folate} + \text{ADP} + P_i$$

H_2-folate is then reduced to H_4-folate by H_2-folate reductase, and H_4-folate cofactors are synthesized in subsequent steps [3]. H_4-folate cofactors donate one carbon units in the biosynthesis of methionine, serine, thymidylate, and purines

To be successful, an antimicrobial agent should inhibit the growth of a

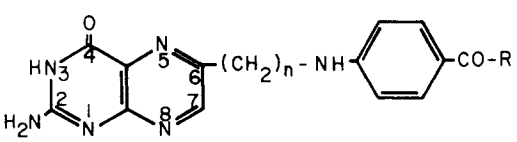
Abbreviations H_2- indicates 7,8-dihydro derivatives, H_4- indicates 5,6,7,8-tetrahydro derivatives See Fig 1

parasitic organism without harming the host The de novo biosynthesis of H_2-folate does not occur in mammals, and thus this pathway is an ideal target for antimicrobial agents Following the formation of H_2-folate, the biosynthetic pathways for the synthesis of H_4-folate and H_4-cofactors are essentially the same in microorganisms and mammals The homofolate and homopteroate antimetabolites previously have been shown to affect the formation and utilization of certain folate cofactors [4,5] However, Kisliuk et al [6] proposed that H_4-homopteroate may affect the biosynthesis of H_2-folate in malaria based on its inhibition of pyrimethamine-resistant *Plasmodium cyanomolgi* That suggestion prompted the investigation of the effects of homopteroates and homofolates on the activity of enzymes involved in H_2-folate biosynthesis Recently [7] we reported on the inhibition of the activity of H_2-pteroate synthetase of *Escherichia coli* and *Plasmodium berghei* by H_2-homopteroate Also, while this manuscript was in preparation, Iwai and Kobashi [8] demonstrated inhibition of the activity of H_2-folate synthetase of *Serratia indica* by H_2-homopteroate

In addition an attempt was made to gain knowledge concerning the metabolic regulation of the biosynthesis of H_2-folate For this reason several related folate and pteroate metabolites were investigated for their effectiveness as inhibitors of this enzyme The activity of H_2-folate synthetase has previously been determined by a microbiological assay with *Lactobacillus casei* [1,2] In order to demonstrate the effects of potential inhibitors on H_2-folate synthetase, an assay utilizing radioactive L-glutamate was developed The radiochemical assay allowed the study of closely related metabolites and antimetabolites which would interfere with the microbiological assay

Materials and Methods

Materials Folic acid and calcium leucovorin were obtained from Lederle Laboratories Division of American Cyanamid Co , *p*-aminobenzoic acid and 2-mercaptoethanol were from Eastman Kodak Co , bovine serum albumin, L-glutamic acid, and Tris were from Sigma Chemical Co , $Na_2S_2O_4$ was from Fisher Scientific, dithiothreitol, ATP, and tetrasodium EDTA were from Cal-Biochem, Sephadex G-100 was from Pharmacia Fine Chemicals, Inc , DEAE Serva Cellulose Ion Exchanger was from Gallard-Schlesinger, amethopterin was



	n	R
PTEROATE	1	OH
HOMOPTEROATE	2	OH
FOLATE	1	GLUTAMATE
HOMOFOLATE	2	GLUTAMATE

Fig 1 Folate structures The dihydro derivatives are reduced at the 7,8 positions and the tetrahydro derivatives are reduced at the 5,6,7 and 8 positions

from Nutritional Biochemicals Corp.; and *p*-aminobenzoylglutamic acid was from Mann Research Laboratories, Inc. Uniformally labeled L-[^{14}C]glutamic acid (260 Ci/mol) was obtained from Schwartz Mann Laboratories Homopteroate and homofolate were obtained from the Drug Development Branch of the National Cancer Institute through the courtesy of Dr Roy Kisliuk, Tufts University, Boston, Mass. Pteroate was a gift from Dr. Charles M Baugh, University of South Alabama, Mobile, Ala.

Purification of H_2-folate synthetase The source of enzyme studied was an *E coli* B resistant to trimethoprim (originally obtained from Dr Robert Greenberg, University of Michigan, Ann Arbor, Michigan) and isolated from a medium containing 128 μg/ml trimethoprim The cells were grown and harvested as described previously [7] Preliminary studies indicated that a 0—50% $(NH_4)_2SO_4$ fraction had sufficient H_2-folate synthetase activity for our use, but it is not known if this is the optimal procedure for purifying this enzyme This fraction was used for the comparison of the radiochemical and the microbiological assays for H_2-folate synthetase

H_2-folate synthetase was further purified by gel filtration chromatography Precipitated protein (30 g) was dissolved in 0 01 M Sorenson's phosphate buffer, pH 7 0 [9], applied to a 5 0 × 90 cm Sephadex G-100 column at 5° C, and eluted with the same buffer The fractions containing H_2-folate synthetase activity were combined The pool had a specific activity of 1 47 nmol/min per mg protein in the H_2-folate synthetase radiochemical assay, a 5-fold increase above the specific activity of the 0—50% $(NH_4)_2SO_4$ fraction The molecular weight of *E coli* B H_2-folate synthetase determined from a single calibrated Sephadex G-200 column was estimated to be 54 000 ± 3000. All kinetic and inhibition studies on H_2-folate synthetase were performed using the Sephadex G-100-purified enzyme

Enzyme assay The activity of H_2-folate synthetase was originally assayed microbiologically following the method of Griffin and Brown [1] except that the total volume of the enzymatic reaction was 0 2 ml and it was found unnecessary to incubate under an atmosphere of hydrogen The reaction mixtures contained 5 mM ATP, 5 mM $MgCl_2$ $6H_2O$, 40 mM KCl, 70 mM 2-mercaptoethanol, 2 mM L-glutamate (sodium), 25 μM H_2-pteroate, 100 mM Tris HCl, pH 8 9 (37° C), enzyme and water to make 200 μl The microbiological assay for H_2-folate was performed with *L casei* (ATCC 7469) maintained in Folic Acid Casei Media (Difco) with 1 ng/ml folic acid Standard curves were obtained with 0—1 ng/ml H_2-folic acid in each assay H_2-folate standards and reaction mixture samples were added prior to autoclaving the medium The inoculum (0 1 ml) consisted of a 6-h culture (10 ml) that had been washed twice with 0 85% NaCl, resuspended in 10 ml of medium, and adjusted to 200 Klett units. The tubes were incubated for 18 h at 37° C and the turbidity measured in a Klett-Summerson photoelectric colorimeter with a 660 nm filter H_2-folate was 50% as effective as folate in supporting the growth of *L casei* This value was not increased when H_2-folate was added aseptically to the sterilized medium in the presence of 0 12% ascorbate

Preparation of pteridines Folate, pteroate, and homofolate were reduced to the corresponding 7,8-dihydro compounds by treatment with sodium dithionite according to the method of Futterman [10] as modified by Blakley [11]

H_2-homopteroate was prepared by reducing homopteroate with $Na_2S_2O_4$ in 2-mercaptoethanol by the method of Friedkin et al [12] All reduced compounds were stored as acidic suspensions in the presence of 0 05—0 2 M dithiothreitol or 2-mercaptoethanol in 0 5 ml samples at —70° C H_4-folate was prepared by the reduction of H_2-folate with *E coli* B H_2-folate reductase and was isolated from a Bio Gel P-2 polyacrylamide gel column H_4-homopteroate, H_4-pteroate and additional H_4-folate were prepared by catalytic hydrogenation following the method of Nahas and Friedkin [13] for the preparation of H_4-homofolate, except that the platinum oxide catalyst was removed by centrifugation and the solution was made 0 11 M with respect to 2-mercaptoethanol following hydrogenation Each reduced compound was characterized by its ultraviolet spectrum [14—16] and by paper chromatography [1,17] Concentrations were determined by ultraviolet spectrum for the dihydro compounds and by chemical formylation [18] for the tetrahydro compounds Chemical reduction to the tetrahydro compounds results in the formation of (±)-isomers and although the effect of having both isomers present in the enzyme assay is not known, similar results were obtained with H_4-folate prepared enzymatically or chemically

H_4-homofolate was prepared by a modification of Kawai and Scrimgeour's [19] method of $Na_2S_2O_4$ reduction A 2-ml solution of 0 4 mM homofolate containing 0 5 M 2-mercaptoethanol was adjusted to pH 7 9 and heated to 50° C in a water bath with continuous stirring $Na_2S_2O_4$ (200 mg) was slowly added and the solution was allowed to sit at 50° C for 30 min, unstirred It was then chilled to 5° C and, while mechanically stirred, the pH was lowered to pH 3 0 with 1 0 M HCl The solution was stored at —70° C and the pH was adjusted to pH 7 0 with dilute NaOH before use H_4-homofolate was characterized by it ultraviolet spectrum [15] and obtained in 88% yield

Analysis of inhibition data The $1/v$ vs $1/s$ plots were analyzed using computer programs for the analysis of competitive and non-competitive inhibitions [20] The apparent K_m values and the V values obtained from the reciprocal plots were analyzed by the student's t-test for statistical significance at the 0 05 level of probability The V values of the inhibited curves differed significantly from that of the uninhibited curve in the inhibition plots termed non-competitive

Miscellaneous A bioautogram was prepared by the method of Brown et al [17] using Folic Acid Casei Media medium containing 3% agar The seed was 1 0 ml of a 10 ml 6 h washed *L casei* culture suspended in 10 ml of 0 85% NaCl Paper chromatograms were developed by descending chromatography in 0 1 M Sorenson's phosphate buffer, pH 7 0 Protein was determined by the method of Lowry et al [21] Radioactive samples were placed in scintillation vials containing 10 ml of a mix of 15 2 g 2,5-bis-2(5-*tert*-butyl-benzoxazolyl)thiopene/gal toluene and counted on a Beckman LS 230 liquid scintillation counter at 75—80% efficiency

Results

Development of radiochemical assay for H_2-folate synthetase

Since the *L casei* assay was not suitable for determining the effects of

inhibitors and metabolites on H_2-folate synthetase, a radiochemical assay method was developed The reaction systems were the same as described in Materials and Methods for the H_2-folate synthetase assay except that in place of non-radioactive L-glutamic acid, 2 mM uniformally labeled L-[^{14}C]glutamic acid (1 25 Ci/mol after dilution with non-radioactive L-glutamic acid) was added The use of L-[^{14}C]glutamic acid allows the quantitation of enzymatically formed H_2-[^{14}C]folate following paper chromatography of the reaction products After incubation, the reactions were stopped by the addition of 50 mM EDTA and placed in ice An aliquot of each reaction (100 μl) and 13 nmol of carrier H_2-folate (10 μl) were applied to Whatman 3MM paper and the chromatograms were developed in 0 1 M Sorenson's phosphate buffer, pH 7.0 L-[^{14}C]glutamic acid moved to the solvent front and the product, H_2-[^{14}C]folate, followed with an R_F of 0 14 Carrier H_2-folate aided in visualizing, under ultraviolet light, the position of the product on the chromatogram This area of fluorescence was cut out and the samples were counted Several observations confirmed that the radioactive area was H_2-[^{14}C]folate Large amounts of enzymatically formed H_2-folate could be visualized under ultraviolet light at an R_F of 0 14 following chromatography of the complete reaction mixture in the absence of carrier H_2-folate Chromatograms of identical reaction mixtures were analyzed for radioactivity and for their ability to support the growth of *L casei* The radioactive tracer scans and the bioautograms of reaction mixtures are seen in Fig 2 The complete reaction mixture had a peak of radioactivity at R_F 0 12, which corresponds to an area of *L casei* growth at R_F 0 12 This peak is absent in the scan of a control reaction mixture lacking H_2-pteroate When a bioautogram of chemically prepared H_2-folate was examined, *L casei* growth was detected at an R_F of approx. 0 16 with a secondary area of growth at an R_F of 0 36 (corresponding to the growth in the presence of folate) Three identical reaction mixtures were assayed mi-

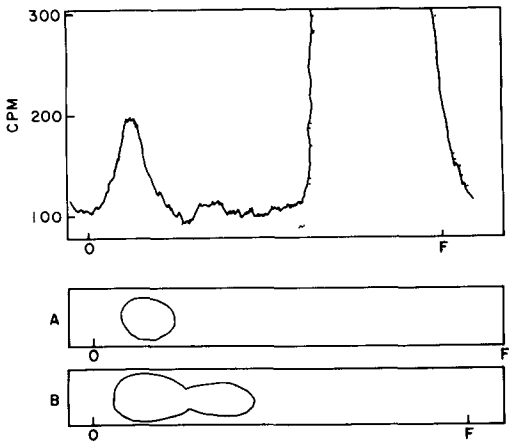


Fig 2 Radioactive tracer scan and bioautograms Chromatograms of a complete H_2-folate synthetase reaction mixture (———) and a control (lacking H_2-pteroate) reaction mixture ( ) were scanned for radioactivity Bioautograms of *L casei* growth in the presence of a complete reaction mixture (A), and a H_2-folate standard (B), indicate that the radioactive product is H_2-folate The origin (O) and front (F) are indicated

crobiologically and in the radiochemical assay The amount of enzymatically formed H_2-folate determined microbiologically (H_2-folate standard curve) was 7 7 μM (range 7 2—8 2 μM) and the amount determined from the radiochemical assay was 7 7 μM (range 6.2—8 6 μM)

There was a linear increase in product formation in the radiochemical assay with increasing protein up to 15 μg (90 min incubation) The apparent K_m value for H_2-pteroate determined from Lineweaver-Burk reciprocal plots was 1 9 μM (0 9—2 3 μM, five experiments)

Inhibition of E coli B H_2-folate synthetase

Table I shows the concentrations of metabolites required for 50% inhibition of *E coli* B H_2-folate synthetase Inhibition was seen with the dihydro derivatives, and the product of the reaction, H_2-folate, was an inhibitor H_4-folate and H_4-homopteroate were at least 5—10 times less effective than the corresponding dihydro derivatives It was estimated by paper chromatography that the dihydro and tetrahydro derivatives were contaminated with less than 10% of pteridines or oxidized metabolites (any contaminent less than 5—10% was difficult to detect). Similar inhibition results were observed testing reduced derivatives of compounds that had been separated from minor fluorescent impurities by DEAE-cellulose chromatography [22] There was no inhibition of *E coli* B H_2-folate synthetase by 2-NH_2-4-OH-6-CH_2 OH-7,8-dihydropteridine (0 59 mM), N^5-formyl H_4-folate (0 73 mM), N^{10}-formyl H_4-folate (0 17 mM), amethopterin (0 025 mM), trimethoprim (4 3 mM), *p*-aminobenzoate (2 5 mM), or *p*-aminobenzoylglutamate (0 35 mM) Although H_2-homopteroate could conceivably be a substrate for this enzyme, the synthesis of H_2-homofolate was not detected Calculation of the minimum amount of product detectable suggested that the reaction proceeded at less than 0.44% the rate of H_2-folate synthetase with an identical amount of H_2-pteroate as substrate Double reciprocal plots (Fig 3) of the inhibition of H_2-folate synthetase by H_2-homopteroate and by the product H_2-folate, with H_2-pteroate as the substrate, showed non-competitive inhibition, since the V of the inhibited curves never reached the V of the control curves The K_i values calculated from the slopes for H_2-homopteroate and H_2-folate were 9 2 ± 2 3 and 23 4 ± 5 9 μM, respectively

TABLE I

INHIBITION OF *E COLI* B DIHYDROFOLATE SYNTHETASE

IC_{50} is the concentration required for 50% inhibition of dihydrofolate synthetase The "greater than" symbol indicates no significant inhibition was observed at the concentration indicated.

Compound	IC_{50} (μM)		
	Aromatic	Dihydro	Tetrahydro
Folate	>2500	116	>700
Homofolate	>385	370	>75*
Pteroate	>790	—	>826
Homopteroate	>2250	136	1280

* The highest level tested due to the limited availability of the compound

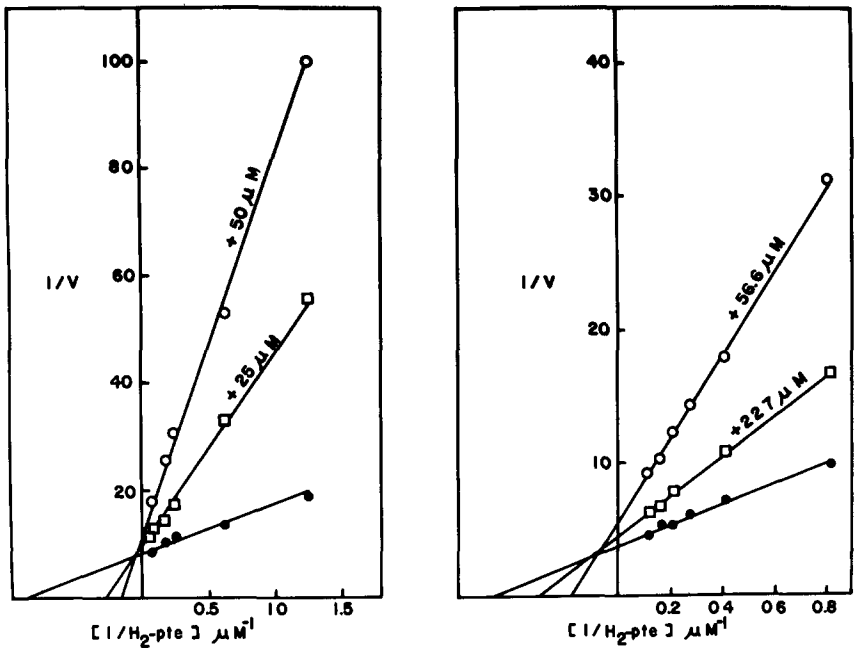


Fig 3 Effects of H_2-homopteroate and H_2-folate on the activity of H_2-folate synthetase from *E coli* B Reciprocal plots of the inhibition by 25 μM (□) and 50 μM (○) H_2-homopteroate, in the left panel, and 22 7 μM (□), and 56 6 μM (○) H_2-folate in the right panel are shown with H_2-pteroate as the varying substrate The control curve (●) has no inhibitor added and the velocity (v) is in units of cpm/14 4 μg protein per 10 min ($\times 10^{-3}$)

Discussion

Since the intracellular pool sizes of H_2-pteroate and H_2-folate are not known, the physiological significance of the product inhibition is uncertain Inhibition of enzymatic activity by low to moderate levels of its product has been reported for dihydroneopterin triphosphate pyrophosphohydrolase [23], dihydroneopterin aldolase [24] and H_2-pteroate synthetase [7] The data presented demonstrates a fourth enzyme in the biosynthesis of H_2-folate in *E coli* that is inhibited by its product The K_i value of each metabolite as a product inhibitor is only 2—5 times higher than the K_m value of that metabolite as a substrate for the next enzyme in the sequence, indicating that inhibitory concentrations of metabolites may be attainable in the cell [25]

Koch [26] proposed that product inhibition of sequential enzymes could regulate the overall rate of the process Although there are many examples of product inhibition that are not concerned with metabolic regulation he considers cofactor biosynthesis an enzymatic sequence potentially subject to this type of control The data discussed in this paper suggests that the biosynthesis of H_2-folate in *E coli* B may be under the influence of metabolic product inhibition

Most previous studies on the mode of action of homofolate and homopteroate derivatives have been concerned with the uptake, utilization, and interconversion of folate cofactors [4,5] The data presented here demonstrates

another site of inhibitory action involving the biosynthesis of H_2-folate, along with the inhibition of H_2-folate synthetase of *S indica* [8], and H_2-pteroate synthetase in *E coli* and of *P berghei* [7] However, these compounds would probably be ineffective growth inhibitors of parasites which synthesize folates de novo, since these organisms usually transport folates poorly [3] Homopteroate (0.3 mM) and H_2-homopteroate (0 093 mM) did not inhibit the growth of *E coli* ML 30 in glucose minimal medium (Warskow, A and Ferone, R , unpublished)

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