Enzyme Studies with New Analogues of Folic Acid and Homofolic Acid*,$

(Received for publication, August 8, 1966)

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SUMMARY

Many new analogues of dihydrofolate and dihydrohomofolic acid have been synthesized and tested as substrates of dihydrofolate reductase. Since all of the dihydro-derivatives were efficiently reduced by the action of dihydrofolate reductase from L1210 mouse leukemia cells, the tetrahydro-derivatives thus formed could be tested directly for cofactor and inhibitor activity with thymidylate synthetase. Although thymidylate synthetase of Escherichia coli showed relatively high sensitivity to changes in the amino acid part of the folate molecule, certain tetrahydro analogues were found to show cofactor activity. These included the aminoadipate, aminopimelate, \( \beta \)-methylglutamate, and lysine analogues of pteroylmonoglutamate. All of the tetrahydrohomofolates inhibited thymidylate synthetase to varying degrees, depending on the nature of the amino acid.

Since June 1962, the goal of this laboratory has been to define more accurately than before the specificity of dihydrofolate reductase and thymidylate synthetase, two enzymes that have a close metabolic relationship. The approach, made possible by the synthesis of many new analogues of pteroylmonoglutamate and homopteroylmonoglutamate, has been a very simple and direct one. All of the dihydrofolate analogues prepared in this study were reduced by the action of dihydrofolate reductase of murine leukemia L1210 cells. Each tetrahydrofolate analogue thus formed could be examined without need of prior isolation for cofactor activity. These included the aminoadipate, aminopimelate, \( \beta \)-methylglutamate, and lysine analogues of pteroylmonoglutamate. All of the tetrahydrohomofolates inhibited thymidylate synthetase to varying degrees, depending on the nature of the amino acid.

EXPERIMENTAL PROCEDURE

Synthesis of Analogues of Folic Acid and Homofolic Acid

Purification of Pteroic Acid

Pteroic acid$ was purified by crystallization of the sodium salt from sodium hydroxide solution. Crude pteroic acid, 10.1 g,

This research was generously supported by United States Public Health Service Research Grant CA-05997 from the National Cancer Institute.

Preliminary reports of this work have been published (1, 2).

We thank Dr. R. B. Angier, Lederle Laboratories, for a gift of sodium pteroate used in the early part of the work. Large amounts of pteroic acid were prepared by Mr. Alan Pratt of this department.

Microanalyses were performed by Dr. S. M. Nagy, Microchemical Laboratories, Massachusetts Institute of Technology, except where otherwise indicated.

Microanalysis by Galbraith Laboratories, Knoxville, Tennessee.

We thank Drs. Leon Goodman and Joseph DeGraw for the details of the synthesis of homofolic acid prior to publication.
406 mg (or 450 mg of N\textsuperscript{31}-trifluoroacetylhomopteroic acid), and 0.20 ml of triethylamine were dissolved in 8 ml of dimethyl formamide by warming to 40\textdegree. The viscous solution was cooled to 20\textdegree and 0.20 ml of isobutyl chloroformate was added. The solution was stirred for 45 min (25-30\textdegree) and 0.40 ml of triethylamine was added followed by 2.5 mmoles of the appropriate amino acid ethyl ester hydrochloride. The mixture was stirred for 20 to 24 hours at 30-35\textdegree and then poured into 150 ml of 0.10 M sodium hydroxide solution. After 90 to 30 min on a boiling water bath under nitrogen, the clear yellow solution was cooled to 15-20\textdegree and adjusted to pH 2 with 12 N hydrochloric acid. The gelatinous analogue was collected by centrifugation at 3000 rpm for 5 to 10 min. The gel was washed three times with twice its volume of distilled water. The gel was then dissolved in the minimum amount of ammonium bicarbonate solution, diluted to 1 l with water, and chromatographed on a column of DEAE-cellulose (3 \times 16 cm). The column was developed with 250 to 500 ml of 0.10 M ammonium bicarbonate and the yellow band was eluted with 0.50 M ammonium bicarbonate. The first yellow band was the folic (or homofolic) analogue.

The ammonium salt of the analogue was usually a gel. The ammonium bicarbonate solution of the desired analogue was evaporated in the flash evaporator (care: frothing; must be protected from bright light) and repeatedly taken down with additional quantities of water (20 to 50 ml) until substantially all of the ammonium bicarbonate had evaporated. The ammonium salt of the analogue was usually a gel. The gel was taken up in 10 ml of 0.10 M sodium hydroxide solution, transferred to a 40-ml centrifuge tube, and centrifuged for 5 min at 3000 rpm. The clear yellow solution was transferred to another tube and the free acid was precipitated by adjusting to pH 3 with 12 N hydrochloric acid. The gelatinous compound was centrifuged at 3000 rpm for 5 min and the supernatant fluid was discarded. The gel was washed by centrifugation three times, each time with a volume of distilled water equal to twice the volume of the gel. After the last centrifugation, the gel was frozen in a bath of Dry Ice-ethanol and lyophilized to dryness. The analytical sample was obtained by drying for 48 hours at 77\textdegree (1 mm Hg) over phosphorus pentoxide. All of the analogues are sensitive to light, but are reasonably stable in the dark in the refrigerator. The analogues were obtained in yields ranging from 100 to 150 mg, with the exception of the p-alanine and \gamma-aminobutyric acid compounds in both series. These were obtained in amounts from 15 to 30 mg. In order to verify the amino acid constituent of these analogues, the synthetic compounds were acid hydrolyzed and chromatographed as follows. Two milligrams of analogue per ml of 3 N HCl in a sealed glass tube were autoclaved for 1.5 hours. Five microliters of hydrolysate were spotted on Whatman No. 3 paper. After ascending chromatography overnight in n-butyl alcohol-acetic acid-H\textsubscript{2}O (100:22:50) (4), the sheet was sprayed with 0.1% ninhydrin in 95% ethanol.

Analytical data\textsuperscript{5} for each of the analogues prepared in this manner follow.

\textit{N-Ptero\textsubscript{2}}-\gamma-aminobutyric Acid—Analysis:

\[ \text{C}_{8\text{H}_{9}}\text{N}_{2}\text{O}_{5} \cdot \text{H}_{2}\text{O} \]

Calculated: C 50.94, H 5.23, N 23.10

Found: C 51.13, H 5.12, N 24.99

\textit{N-Ptero\textsubscript{2}-\textalpha-}aminoadipic Acid—Analysis:

\[ \text{C}_{8\text{H}_{9}}\text{N}_{2}\text{O}_{5} \cdot \text{H}_{2}\text{O} \]

Calculated: C 54.79, H 5.01, N 20.32

Found: C 54.99, H 4.93, N 19.93

\textit{N-Ptero\textsubscript{2}-\textalpha-}aminopimelic Acid—Analysis:

\[ \text{C}_{8\text{H}_{9}}\text{N}_{2}\text{O}_{4} \cdot 2\text{H}_{2}\text{O} \]

Calculated: C 49.89, H 5.39, N 19.40

Found: C 50.05, H 5.50, N 19.50

\textit{N-Ptero\textsubscript{2}-\textalpha-}methylglutamic Acid—Analysis:

\[ \text{C}_{8\text{H}_{9}}\text{N}_{2}\text{O}_{4} \cdot \text{H}_{2}\text{O} \]

Calculated: C 50.73, H 4.90, N 20.71

Found: C 50.54, H 5.08, N 21.03

\textit{N-Ptero\textsubscript{2}-\textgamma-\textbeta-methylglutamic Acid—Analysis:

\[ \text{C}_{8\text{H}_{9}}\text{N}_{2}\text{O}_{4} \cdot 2\text{H}_{2}\text{O} \]

Calculated: C 48.87, H 4.51, N 19.94

Found: C 48.37, H 4.75, N 19.94

\textit{N-Ptero\textsubscript{2}-\textgamma-\textgamma-hydroxy glutamic Acid—Analysis:

\[ \text{C}_{8\text{H}_{9}}\text{N}_{2}\text{O}_{4} \cdot \text{H}_{2}\text{O} \]

Calculated: C 48.49, H 4.45, N 20.62

Found: C 47.74, H 4.39, N 20.34

\textit{N-Homopteroylglycine—Analysis:

\[ \text{C}_{8\text{H}_{9}}\text{N}_{2}\text{O}_{4} \cdot \text{H}_{2}\text{O} \]

Calculated: C 47.90, H 4.50, N 23.32

Found: C 47.91, H 4.73, N 23.49

\textit{N-Homopteroylglycine—Analysis:

\[ \text{C}_{8\text{H}_{9}}\text{N}_{2}\text{O}_{4} \cdot \text{H}_{2}\text{O} \]

Calculated: C 52.03, H 4.62, N 24.99

Found: C 52.65, H 4.54, N 24.17

\textit{N-Homopteroylglycine—Analysis:

\[ \text{C}_{8\text{H}_{9}}\text{N}_{2}\text{O}_{4} \cdot \text{H}_{2}\text{O} \]

Calculated: C 50.84, H 4.68, N 22.77

Found: C 51.83, H 4.50, N 22.83

\textit{N-Homopteroylglycine—Analysis:

\[ \text{C}_{8\text{H}_{9}}\text{N}_{2}\text{O}_{4} \cdot \text{H}_{2}\text{O} \]

Calculated: C 50.05, H 4.86, N 22.21

Found: C 50.81, H 4.86, N 22.21

\textsuperscript{5} Microanalysis by Schwartzkopf Microanalytical Laboratory, Woodside, New York.
New Analogues of Folic Acid and Homofolic Acid

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Preparation of Lysine Analogues of Folic Acid and Homofolic Acid

\[ N^\text{-Homopteryl-L(+)} \text{-aspartic Acid—Analysis:} \]
\[ C_{12}H_{16}N_{2}O_{4} \cdot H_{2}O \]
Calculated: C 48.71, H 4.74, N 20.93
Found: C 49.19, H 4.44, N 20.70

\[ N^\text{-Homopteryl-\text{-}N\text{-}} \text{-glutamic Acid—Analysis:} \]
\[ C_{13}H_{18}N_{2}O_{4} \cdot H_{2}O \]
Calculated: C 51.73, H 4.78, N 21.11
Found: C 52.14, H 4.70, N 21.69

\[ N^\text{-Homopteryl-DL-\alpha\text{-}aminoadipic Acid—Analysis:} \]
\[ C_{17}H_{22}N_{4}O_{4} \]
Calculated: C 51.74, H 5.17, N 20.12
Found: C 51.01, H 4.92, N 20.54

\[ N^\text{-Homopteryl-DL-\alpha\text{-}methylglutamic Acid—Analysis:} \]
\[ C_{16}H_{16}N_{3}O_{4} \]
Calculated: C 53.65, H 5.32, N 19.91
Found: C 53.80, H 5.19, N 20.32

\[ N^\text{-Homopteryl-DL-\alpha\text{-}methylglutamic Acid—Analysis:} \]
\[ C_{16}H_{16}N_{3}O_{4} \]
Calculated: C 53.72, H 4.94, N 20.89
Found: C 53.51, H 4.94, N 21.08

\[ N^\text{-Homopteryl-DL-DL-\alpha\text{-}methylglutamic Acid—Analysis:} \]
\[ C_{17}H_{22}N_{4}O_{4} \]
Calculated: C 51.74, H 4.95, N 20.12
Found: C 51.54, H 4.95, N 20.37

\[ N^\text{-Homopteryl-DL-DL-\alpha\text{-}methylglutamic Acid—Analysis:} \]
\[ C_{17}H_{22}N_{4}O_{4} \]
Calculated: C 52.71, H 5.06, N 20.49
Found: C 52.29, H 5.28, N 20.41

\[ N^\text{-Homopteryl-DL-DL-\alpha\text{-}methylglutamic Acid—Analysis:} \]
\[ C_{17}H_{22}N_{4}O_{4} \]
Calculated: C 50.00, H 4.62, N 20.41
Found: C 50.34, H 4.52, N 20.67

\[ N^\text{-Homopteryl-DL-DL-\alpha\text{-}hydroxyglutamic Acid—Analysis:} \]
\[ C_{17}H_{22}N_{4}O_{4} \]
Calculated: C 50.10, H 4.84, N 22.37
Found: C 50.55, H 4.71, N 23.30

Preparation of Lysine Analogues of Folic Acid and Homofolic Acid

\[ N^\text{-Acetyl-N\text{-}N\text{-}trifluoroacetylpteroyl-} \text{aspartic Acid,} \] 450 mg (or 494 mg of \[ N^\text{-Acetyl-N\text{-}N\text{-}trifluoroacetylhomopteroic acid,} \] was dissolved in 8 ml of dimethylformamide containing 0.20 ml of triethylamine. The solution was cooled to 10–15°C, and 0.20 ml of isobutyl chloroformate was added. The mixture was stirred at 30°C for 45 min, and 743 mg of \[ N^\text{-N\text{-trifluoroacetylamino}-} \text{valeric acid-L(+)} \text{-lysine methyl ester hydrochloride} \] were added. After stirring for 24 hours at 30–35°C, the mixture was evaporated to dryness, washed with 20 ml of water two times, filtered, and dried. The mixture was washed with 20 ml of ether three times, filtered, and dried. The dry mixture (520 to 550 mg) was dissolved in 10 ml of dimethylformamide and chromatographed on a column (2.5 x 7.5 cm) of DEAE-cellulose in dimethylformamide. The fully blocked \[ N^\text{-N\text{-trifluoroacetylamino}-} \text{valeric acid-L(+)} \text{-lysine methyl ester hydrochloride} \] was washed through with dimethylformamide, leaving the blocked pteric or homopteroic acid on the column. The eluate was evaporated to dryness and the residue was washed with ether and dried, giving approximately 400 mg of pure fully blocked compound.

Hydrolysis (100 mg of compound in 50 ml of 0.20 N sodium hydroxide, under nitrogen at 100°C for 45 min) gave \[ N^\text{-N\text{-trifluoroacetylamino}-} \text{valeric acid-L(+)} \text{-lysine} \].

\[ C_{19}H_{23}N_{3}O_{4} \]
Calculated: C 54.63, H 6.05, N 20.39
Found: C 54.41, H 5.72, N 20.40

\[ N^\text{-Homopteryl-N\text{-}N\text{-trifluoroacetylamino}-} \text{valeric acid-L(+)} \text{-lysine—Analysis:} \]
\[ C_{19}H_{23}N_{3}O_{4} \]
Calculated: C 53.70, H 6.41, N 19.27
Found: C 53.35, H 5.82, N 18.67

The \[ N^\text{-N\text{-trifluoroacetylamino}-} \text{valeric acid-L(+)} \text{-lysine} \] group was removed by suspending 100 mg of compound in 25 ml of 2 N HCl. After stirring for 1 hour at 25–30°C, the solution was cooled to 10–15°C, adjusted to pH 3, and centrifuged at 3000 rpm for 5 min. The gel was washed by suspension once with 10 ml of water, centrifuged, and lyophilized to dryness. On further washing, the compounds peptized and could not be centrifuged. Samples free of sodium chloride were not obtained, but each compound traveled as a single spot on paper chromatography.

Chromatographic Behavior of Analogues

In Table I are collected the data for the movement on paper of each analogue in three aqueous salt solutions.

Preparation of Dihydrofolate Analogues

The dihydrofolic analogues of folic acid and homofolic acid were prepared by reduction with sodium dithionite at room temperature either by the procedure of Futterman (6), in volving precipitation from an acidified medium containing ascorbate, or by a modification (7) wherein the reduced dihydrofolic acid precipitates spontaneously from a medium containing mercaptoethanol. The spectra of all the dihydrofolate analogues in 0.1 N NaOH and 0.05 M Tris-chloride buffer, pH 7.4, closely resembled that of dihydrofolic acid. Usually, the reduction of 5–10 mg amounts of each analogue yielded enough dihydro-compound for kinetic studies.

Reduction in Medium Containing Ascorbate—Five milligrams of folic acid or folic acid analogue were dissolved in 0.5 ml of H2O with a minimum amount of N NaOH. An equal volume of ascorbic acid solution (200 mg of ascorbic acid per ml of H2O, adjusted to pH 6, with N KOH) was then mixed with the folate. At this point or upon addition of sodium dithionite, some of the folate analogues precipitated because of varying solubilities. A minimum amount of N NaOH was added to clarify the solution. The color change from bright to pale yellow was used as a rough index of reduction. In most cases, this occurred within 15 min at room temperature; however, in some solutions the color persisted longer than 15 min. Some solutions even darkened initially. In these cases, the reduction step was allowed to continue at room temperature for 1 to 2 hours. The dihydro-
folate analogues were then precipitated by adjusting chilled solutions to pH 2 to 4 by the slow addition of 2 ml of 0.05 M Tris-chloride buffer, pH 7.4, containing 0.001 M sodium versenate and 0.01 M mercaptoethanol; the final pH was usually about 4.

Reduction in Medium Containing Mercaptoethanol—Twelve milligrams of folic acid or folic acid analogue were dissolved in 1 ml of 1 M mercaptoethanol by the minimal addition of 0.05 M HCl. Each dihydrofolate analogue was stored as a suspension in 0.5 ml of 0.05 M Tris-chloride buffer, pH 7.4, containing 0.001 M sodium versenate and 0.01 M mercaptoethanol; the final pH was usually about 4.

The mixture was gently swirled at room temperature over a period of several minutes. After a precipitate had formed, the suspension was transferred to a small tube, chilled, and centrifuged. The precipitate was washed once by suspension in 1 ml of 1 M mercaptoethanol and twice with 1 ml portions of 0.01 M mercaptoethanol. The dihydrofolate analogue was stored as a suspension in 0.01 M mercaptoethanol.

In this modification of Futterman’s procedure, there is usually no need to acidify the reaction mixture since a drop in pH occurs during reduction of the folate with dithionite. The decrease in pH depends in part on oxidation of dithionite by dissolved oxygen. If the reaction is carried out under anaerobic conditions, the drop in pH is not sufficient for precipitation of dihydrofolic acid. Thus the amount of shaking, the rate of diffusion of air into the reaction mixture, and the ratio of surface area to volume all influence the course of the reaction.

Enzyme Preparations

Dihydrofolate Reductase

The source of dihydrofolate reductase was an extract prepared from spleens and tumors of male mice (CDBA, National Institutes of Health animal farm) implanted with amethopterin-resistant (FR8 and FR9) lymphoid leukemia L1210 cells (8). These cells have high levels of dihydrofolate reductase. In a typical preparation, acetone powder (1.6 g) was extracted with 16 ml of 0.1 M potassium phosphate buffer, pH 7.5, and the nucleic acids were removed by precipitation with 0.3 volume of 2% protamine sulfate. From the supernatant fluid thus obtained dihydrofolate reductase was precipitated between 50 and 75% saturated ammonium sulfate.

Assays with Dihydrofolate Reductase

Sufficient dihydrofolic acid or dihydrofolic acid analogue was added to 1 ml of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.08 mM TPNH to give an initial total absorbance of 0.7 at 340 m\(\mu\); then enough dihydrofolate reductase was added to give an absorbance decrease of 0.02 to 0.04 per min at 37\(^\circ\) with dihydrofolic acid. The initial rate of reduction of the dihydrofolate analogue was compared with dihydrofolic acid as the standard.

Thymidylate Synthetase

E. coli R cells, purchased from a commercial source (Grain Processing Corporation, Muscatine, Iowa), were disrupted by grinding with glass beads in a Waring Blender. The viscous extract was then subjected to fractionation procedures involving the removal of nucleic acids with streptomycin sulfate, precipitation with ammonium sulfate, and chromatography on DEAE-cellulose followed by chromatography on Sephadex G-100 (9). The purified synthetase can be stored at -10\(^\circ\) for months with negligible loss of activity if 30 to 60% sucrose is present.

Standard Assay of Thymidylate Synthetase

The spectrophotometric assay of thymidylate synthetase based on the oxidation of tetrahydrofolate to dihydrofolic acid was used routinely (10).

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>(% J_{160}^{A})</th>
<th>(% J_{160}^{B})</th>
<th>(% J_{160}^{C})</th>
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</thead>
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<tr>
<td>N-Pteroyl-L(+)-glutamic acid (folic acid)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
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<td>1.00</td>
<td>1.00</td>
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<tr>
<td>N-Homopteroyl-L(-)-glutamic acid</td>
<td>1.27</td>
<td>1.69</td>
<td>1.69</td>
</tr>
<tr>
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<td>0.19</td>
<td>0.16</td>
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<td>N-Pteroylsalanine</td>
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<td>0.89</td>
<td>0.82</td>
</tr>
<tr>
<td>N-Homopteroylsalanine</td>
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<td>0.26</td>
<td>0.23</td>
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<tr>
<td>N-Pteroyl-L(-)-alanine</td>
<td>0.89</td>
<td>0.92</td>
<td>0.79</td>
</tr>
<tr>
<td>N-Homopteroyl-L(-)-alanine</td>
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<td>0.46</td>
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<td>N-Homopteroyl-DL-alanine</td>
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<td>0.95</td>
<td>0.85</td>
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<td>0.27</td>
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<tr>
<td>N-Homopteroyl-(\gamma)-aminobutyric acid</td>
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<td>0.89</td>
<td>0.82</td>
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<tr>
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<td>1.82</td>
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<td>N-Homopteroyl-DL-a-aaminoacidic acid</td>
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<td>1.29</td>
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<td>N-Pteroyl-DL-DL-methylglutamic acid</td>
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<td>N-Pteroyl-DL-DL-DL-(\beta)-methylglutamic acid</td>
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<td>N-Homopteroyl-DL-DL-DL-(\gamma)-aminoglutamic acid</td>
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<td>0.89</td>
<td>0.86</td>
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<tr>
<td>N-Pteroyl-DL-DL-DL-(\gamma)-hydroxyglutamic acid</td>
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<td>1.00</td>
<td>1.00</td>
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<td>N-Homopteroyl-DL-DL-(\gamma)-hydroxyglutamic acid</td>
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<td>1.71</td>
<td>1.73</td>
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<tr>
<td>N(^8)-Pteroyl N(6)-t-butylxoycarbonyl L(+)-lysine</td>
<td>0.92</td>
<td>1.07</td>
<td>0.97</td>
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<tr>
<td>N(^8)-Homopteroyl-N(6)-t-butylxoycarbonyl L(+)-lysine</td>
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<td>1.35</td>
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<tr>
<td>N(^8)-Pteroyl-L(+)-lysine</td>
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<tr>
<td>N(^8)-Homopteroyl-L(+)-lysine</td>
<td>0.84</td>
<td>1.32</td>
<td>1.30</td>
</tr>
<tr>
<td>Pteric acid</td>
<td>0.26</td>
<td>0.29</td>
<td>0.27</td>
</tr>
<tr>
<td>Pteric acid</td>
<td>0.75</td>
<td>0.83</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* Chromatography was on Whatman No. 1 paper ascending. Solvents were: A, 0.1 N NH\(4\)HCO\(3\); B, 5% Na\(2\)HPO\(4\); C, 4% sodium citrate.

* Distance traveled is expressed as fraction of distance travelled by folic acid (N-pteroyl-L(+)-glutamic acid).

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approximately 0.02 to 0.04 mM), dihydrofolate reductase with an inhibitory thus formed with thymidylate synthetase. The complete thesis proceeded during this period. The small absorbance in-crease was possible. Occasionally, with less pure preparations for the complete mixtures.

cuvettes. The absorbance rise in Cuvette 3 indicated that thymidylate synthetase was added to each of the three cuvettes. In Cuvette 2, thymidylate synthetase was added to each cuvette. Since no absorbance increase occurred in Cuvette 3, this indicated complete inhibition of thymidylate synthetase by tetrahydrohomopteroyl-α-aminopimelate.

It is important to stress that the nonenzymic reaction of formaldehyde with the tetrahydropteroyl derivatives is not limited in these studies, despite the presence of mercaptoethanol and Tris buffer. Under conditions described in the legend of Fig. 1, equilibration of the tetrahydro-analogues with formaldehyde occurs within 1 to 2 min. Preincubation of formaldehyde with the tetrahydropteroyl analogues did not yield higher thymidylate synthetase activities. Furthermore, a 4-fold in-

FIG. 1. The enzymatic reduction of N-dihydropteroyl-β-L-aminopimelate to N-tetrahydropteroyl-β-L-aminopimelate and demonstration of the cofactor activity of the tetrahydro- derivative thus formed with thymidylate synthetase. The complete reaction mixture contained sufficient N-dihydropteroyl-β-L-aminopimelate to give an absorbance of 0.2 to 0.4 at 340 nm (approximately 0.02 to 0.04 mM), dihydrofolate reductase with an activity to yield an absorbance decrease of 0.02 to 0.04 at 340 nm within a 3- to 10-min period, 0.02 to 0.03 mM TPNH, 0.012 mM formaldehyde, 0.04 mM DUMP, 0.09 mM mercaptoethanol, 0.02 mM MgCl₂, and 0.04 mM Tris-HCl (pH 7.4). A similar mixture containing dihydrofolate was prepared as a control. Period A, dihydrofolate was reduced to tetrahydrofolate in control Cuvette 1. During the same period, dihydropteroyl-α-aminopimelate was added to tetrahydropteroyl-β-aminopimelate in Cuvette 2 (dUMP omitted) and Cuvette 3 (complete mixture). In all three cuvettes, the absorbance decrease was due to the oxidation of TPNH and the concomitant conversion of the dihydro- form to the tetrahydro- compound. Period B, amethopterin (1 X 10⁻⁸ M) was added to each of the three cuvettes. This concentration inhibits dihydrofolate reductase but not thymidylate synthetase. Period C, thymidylate synthetase was added to each of the three cuvettes. The absorbance rise in Cuvette 3 indicated that thymidylate synthetase had occurred with tetrahydropteroyl-α-aminopimelate, whereas in the absence of dUMP (Cuvette 2) no increase was possible. Occasionally, with less pure preparations of thymidylate synthetase, some absorbance increase due to turbidity occurred in Cuvette 2 during Period C; in this case, the absorbance increases in Cuvettes 1 and 3 were corrected accordingly. Cuvette 1, containing tetrahydrofolate, served as a control for Period C, indicating that conditions were right for thymidylate synthesis. Period D, dl-β-tetrahydrofolate (0.15 mM) was added to each of the cuvettes. In Cuvettes 1 and 3, thymidylate synthesis proceeded during this period. The small absorbance increase in Cuvette 2 (dUMP absent) was used to correct the values for the complete mixtures.

General Method Used for Testing Potential Cofactor and Inhibitory Activity of Tetrahydrofolate Analogues with Thymidylate Synthetase

**Basis of Procedure**

Each dihydrofolate analogue was converted enzymically to the tetrahydrofolate analogue with dihydrofolate reductase and TPNH. Amethopterin was then added to inhibit dihydrofolate reductase during the ensuing period (11). Thymidylate synthetase was added to test for potential cofactor activity of the tetrahydrofolate analogue. Tetrahydrofolate was subsequently added to test for possible inhibitory properties of the tetrahydrofolate analogue.

Details for this general procedure are presented in the legend of Fig. 1, with dihydropteroyl-β-L-aminopimelate as an example. This analogue was readily reduced enzymically to the tetrahydro- derivative, and the tetrahydro- compound thus formed had good cofactor activity, i.e. 63% relative to tetrahydrofolate.

In contrast to the above findings, when dihydrohomopteroyl-β-L-aminopimelate was reduced enzymically to the tetrahydro-derivative, the tetrahydro- compound was not a cofactor of thymidylate synthetase; indeed it markedly inhibited the synthetase (Fig. 2).

It is important to stress that the nonenzymic reaction of formaldehyde with the tetrahydropteroyl derivatives is not limited in these studies, despite the presence of mercaptoethanol and Tris buffer. Under conditions described in the legend of Fig. 1, equilibration of the tetrahydro-analogues with formaldehyde occurs within 1 to 2 min. Preincubation of formaldehyde with the tetrahydropteroyl analogues did not yield higher thymidylate synthetase activities. Furthermore, a 4-fold in-

FIG. 2. The enzymatic reduction of N-dihydrohomopteroyl-β-L-aminopimelate to N-tetrahydrohomopteroyl-β-L-aminopimelate and demonstration of the inhibition of thymidylate synthetase by the tetrahydro derivative thus formed. The procedure followed was identical with that described in the legend of Fig. 1. Period A, dihydrofolate was reduced to tetrahydrofolate in control Cuvette 1. During the same period, dihydrohomopteroyl-α-aminopimelate was added to tetrahydrohomopteroyl-α-aminopimelate in Cuvette 2 (dUMP omitted) and Cuvette 3 (complete mixture). Period D, amethopterin (1 X 10⁻⁸ M) was added to each cuvette. Period C, thymidylate synthetase was added to each cuvette. Since no absorbance increase occurred in Cuvette 3, this indicated that tetrahydrohomopteroyl-α-aminopimelate was not a cofactor of thymidylate synthetase. Period D, dl-β-tetrahydrofolate (0.15 mM) was added to each cuvette. Since no absorbance increase occurred in Cuvette 3, this indicated complete inhibition of thymidylate synthetase by tetrahydrohomopteroyl-α-aminopimelate.
crease of formaldehyde to a final concentration of 0.05 m also did not yield increased enzyme rates.

Similarly, with the tetrahydrohomopteroyl derivatives, preincubation before addition of thymidylate synthetase or the presence of higher concentrations of formaldehyde did not enhance the inhibitory action of the tetrahydrohomopteroyl derivatives.

RESULTS

Specificity of Dihydrofolate Reductase—Dihydrofolate reductase catalyzes the reduction of a large variety of 6-substituted derivatives of 2-amino-4-hydroxy-7,8-dihydropteridine. In Tables II and III are listed the relative rates of reduction observed in the present study with analogues of dihydrofolate and dihydrohomofolate.

The most pronounced effect of altering the structure of dihydrofolate was found with halogenated compounds. A 3'-iodo substituent in dihydrofolate or dihydrohomofolate gave a 4- to 6-fold exaltation of the enzymatic rate of reduction, an increase that was also observed with 3',5'-dibromohydrofolate. In contrast to the enhancing effects of halogenation, the introduction of a nitrosoamine structure between the pteridine and benzene rings resulted in a sharp depression of the rate of enzymatic reduction, as shown in the lower part of Table II. These compounds were reduced at one-fourth to one-fifth the rate of the secondary amino compound from which they were prepared. This effect was strong enough to overcome the rate enhancement observed in the halogenated dihydrofolates.

Dihydrofolate reductase tolerates wide variations in structure in the glutamate part of folate (Table III). Since N-dihydro-

### Table III

**Enzymatic reduction of dihydropteroyl and dihydrohomopteroyl amino acids**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Relative rate</th>
<th>N-Dihydropteroyl</th>
<th>N-Dihydrohomopteroyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>L(+) -Glutamate</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>D(−) -Glutamate</td>
<td>0.53</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>D-L- α-Methylglutamate</td>
<td>0.36</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>D-L- β-Methylglutamate</td>
<td>1.0</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>D-L-γ-Methylglutamate</td>
<td>1.2</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>D-L-β-Hydroxyglutamate</td>
<td>0.59</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>D-L-γ-Aminoglutamate</td>
<td>0.35</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>L(+)-Aspartate</td>
<td>0.42</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>D-L-α-Aminopimelate</td>
<td>0.83</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>D-L-α-Aminopimelate</td>
<td>1.9</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>N*-L(+) -Lysine</td>
<td>1.0</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>N*- (β-Butyloxycarbonyl)-N*-L(+) -Lysine</td>
<td>1.5</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>1.20</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>β-Alanine</td>
<td>0.36</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>γ-Aminobutyrate</td>
<td>0.33</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>D-L-Alanine</td>
<td>1.10</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>α-Glutamyl-γ-glutamyglutamate</td>
<td>1.30</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>γ-Glutamyl-glutamyglutamate</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydropteroyl</td>
<td>0.27</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Dihydrohomopteroyse</td>
<td>0.27</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

* We thank Dr. R. B. Angier, Lederle Laboratories, for gifts of these compounds.

† We thank Dr. Alton Meister for gifts of the three methyl glutamic acids.

pteroyl-d(−)-glutamate can be reduced at one-half the rate of the substrate containing L(+)-glutamate, stereospecificity at the α carbon of glutamate is not stringently defined. A similar situation was observed with the two stereoisomers in the dihydrohomopteroyl series of compounds.

It should be noted that the relative rates obtained with methyl, amino, and hydroxy derivatives of racemic glutamic acid (Table III) must result from a composite of rates represented by each of the possible isomers present. Nevertheless, certain deductions can be made.

The relatively low degree of selectivity for the α-carboxyl region of glutamate is shown by the various methyl derivatives of glutamate. Despite the introduction of the hydrophobic and sterically demanding methyl group at the α position of glutamate, relative rates of 0.36 and 0.40 were observed for N-dihydropteroyl- and N-dihydrohomopteroyl-α-methyl glutamates, respectively. Introduction of methyl groups more distant from the α position, as exemplified by the β- and γ-methyl glutamates, resulted in substrate activities equivalent to unsubstituted L(+)-glutamate. Indeed, these compounds, with the exception of N-dihydrohomopteroyl-β-methyl glutamate, were reduced at faster rates than that expected for a mixture containing racemic glutamate, indicating that hydrophobic character in the vicinity of the γ-carboxyl of glutamate is a favorable substrate property. Interestingly, the hydrophilic γ-amino and γ-hydroxyglutamates showed a falling off in relative rates of reduction when compared to the γ methyl glutamates.

The apparently favorable substrate property of hydrophobic
TABLE IV

<table>
<thead>
<tr>
<th>Tetrahydro- compounds as cofactors for thymidylate synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Tetrahydropteroyl-</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>Glycine...</td>
</tr>
<tr>
<td>L-Aspartate...</td>
</tr>
<tr>
<td>L-Glutamate...</td>
</tr>
<tr>
<td>D(-)-Glutamate...</td>
</tr>
<tr>
<td>DL-L-Aspartate...</td>
</tr>
<tr>
<td>DL-L-Aminopimelate...</td>
</tr>
<tr>
<td>DL-L-Methylglutamate...</td>
</tr>
<tr>
<td>DL-DL-L-Aspartate...</td>
</tr>
<tr>
<td>DL-DL-L-Methylglutamate...</td>
</tr>
<tr>
<td>DL-DL-L-Hydroxyglutamate...</td>
</tr>
<tr>
<td>DL-DL-L-Aminoglutarate.</td>
</tr>
<tr>
<td>L-Glutamate...</td>
</tr>
<tr>
<td>L-Aspartate...</td>
</tr>
<tr>
<td>Tetrahydropteroyl...</td>
</tr>
<tr>
<td>Tetrahydrohomopteroate</td>
</tr>
<tr>
<td>Tetrahydrobis-homopteroate</td>
</tr>
</tbody>
</table>

* Each analogue was tested under conditions exactly as described in the legend of Fig. 1.
* To confirm results obtained with enzymically generated tetrahydrofolate analogues, dithionite reduction (as described by Silverman and Noronha (15)) for the preparation of tetrahydrofolate) was used. The tetrahydrofolate analogue was purified by gradient elution from DEAE-cellulose (0.005 M Tris-HCl buffer (pH 7.4), 0.2 M mercaptoethanol (0 to 1 M NaCl)). The tetrahydrofolate analogue thus obtained was tested for cofactor activity in the standard thymidylate synthetase system. The values obtained for relative cofactor activities when the tetrahydro-analogues were prepared in this way showed good agreement with the values obtained for the enzymically generated compound.
* These tetrahydrofolate analogues were also prepared by catalytic reduction. The relative cofactor activities obtained for compounds prepared by this method showed good agreement with the values obtained as in Footnotes a and b.
* To confirm results obtained with enzymically generated tetrahydrofolate analogues, dithionite reduction (as described by Silverman and Noronha (15)) for the preparation of tetrahydrofolate) was used. The tetrahydrofolate analogue was purified by gradient elution from DEAE-cellulose (0.005 M Tris-HCl buffer (pH 7.4), 0.2 M mercaptoethanol (0 to 1 M NaCl)). The tetrahydrofolate analogue thus obtained was tested for cofactor activity in the standard thymidylate synthetase system. The values obtained for relative cofactor activities when the tetrahydro-analogues were prepared in this way showed good agreement with the values obtained for the enzymically generated compound.

STRUCTURE 1

The activities of the last 12 compounds listed in Table III re-emphasize the generally low substrate specificity of dihydrofolate reductase. Those analogues containing no L-carboxyl and pteroyl-α-glutamyl glutamate (in which the α-carboxyl is blocked) were reduced at rates not much higher than dihydropteroate which contains no amino acid at all. The glycine and Nα-alanine compounds showed good substrate behavior, with the exception of N-dihydrohomopteroyl-glycine.

Specificity of Thymidylate Synthetase—In contrast to the low level of selectivity of dihydrofolate reductase, thymidylate synthetase shows considerably more stringent structural requirements for analogues with cofactor activity. Although halogenated analogues of dihydrofolate were reduced at faster rates than dihydrofolate (Table II), the resulting halogenated tetrahydrofolates were essentially inactive as cofactors of thymidylate synthetase (Table IV). Since spectral shifts observed when formaldehyde was mixed with either 3',5'-dibromotetrahydrofolate or with 3'-iodotetrahydrofolate were comparable to that observed with tetrahydrofolate, it was concluded that the cofactor inactivity of these analogues could not be attributed to lack of formaldehyde binding.

Various tetrahydropteroyl amino acids with and without cofactor activity are compared in Table IV. Thymidylate synthetase is sensitive to changes in the vicinity of the α-carboxyl of analogues with potential cofactor activity. The stereoisomers tetrahydropteroyl-(−)-glutamate showed slight activity, if any. The L-alanine and γ-aminoantibutyric acid analogues, lacking the α-carboxyl, were completely inactive, as was tetrahydropteroyl-α-glutamyl glutamate, which contains a blocked α-carboxyl group. The glycine and Nα-alanine compounds, lacking the carbon skeleton of glutamate, showed no cofactor activity. Introduction of the hydrophobic methyl group at the sensitive α position of glutamate destroyed the cofactor activity completely. When the methyl group was moved to the α or γ position of glutamate, the analogues were able to take part in the enzymatic synthesis of thymidylate, but only at a rate about one-third to one half that of tetrahydrofolate. The hydrophilic γ-hydroxyglutamate also caused a sharp decrease in the rate of the reaction; with the γ-amino compound, the rate fell to zero.

The aminodicarboxylic acid derivatives (aspartate, amino-adipate, and aminopimelate) showed varying degrees of cofactor activity. Tetrahydropteroyl-(−)-aspartate was relatively poor compared to tetrahydrofolate. Although the two higher homologues of glutamate (aminoadipate and aminopimelate) showed the best activity of all of the analogues studied, they were still below the level of tetrahydrofolate. In view of the existence of naturally occurring polyglutamate forms of the folates, tetrahydropteroyl-γ-glutamyl-γ-glutamyl-glutamate was tested. It showed no greater activity than the monoglutamate form of folate.

Nα-Tetrahydropteroyl-(−)-lysine showed an unexpectedly high level of cofactor activity. Apparently the sensitivity of thymidylate synthetase to changes in the glutamate part of the molecule does not extend to the terminal position, because the enzyme can tolerate a change in ionic charge in this region.

Three of the compounds showing intermediate cofactor activity were subjected to more detailed kinetic investigation in order to
determine whether the relatively lower cofactor activities of these analogues compared to tetrahydropteroyl-β-glutamate were due to a decrease in affinity (i.e. a higher $K_m$) or to an intrinsically lower activity. The maximal velocity attainable with saturating amounts of the β-methylglutamate derivative was 50% that of folate; with γ-hydroxyglutamate the value was 12.5% of folate; and with the N°-lysine derivative, 44% (Table V). Although it is not easy to make comparisons with analogues consisting of several isomers, the following considerations have some validity. Those compounds, containing 3 asymmetrical carbon atoms, yield eight possible isomers; however, only one configuration at carbon 6 and only the L configuration at the α-amino carbon should allow cofactor activity. Thus only two of the eight isomers should be active. The data in Table V are consistent with the following: two of eight isomers of β-methyl glutamate and of γ-hydroxyglutamate and one isomer of the lysine analogue have affinities that are of the same order of magnitude as that of tetrahydropteroyl-L(+)-glutamate. It is concluded that the relatively low cofactor activity of the methyl glutamate, hydroxyglutamate, and lysine derivatives was due to decreased affinity for thymidylate synthetase but to less intrinsic activity.

Inhibition of Thymidylate Synthetase—Tetrahydrohomofolate is a fairly potent inhibitor of thymidylate synthetase (1). The effects of further modifying this inhibition were studied by preparing a series of amino acid derivatives. As shown in Table VI, the presence of the additional methylene group (α°) in homopteroyl compounds is sufficient to cause some inhibition of thymidylate synthetase by all of the analogues tested. Those compounds lacking the carbon skeleton of glutamate (glycine, β-alanine, γ-aminobutyrate, and DL-alanine) inhibited the enzymatic reaction at a level of one-fifth or lower than the inhibitory level of tetrahydrohomofolate. Surprisingly, the requirement of the L(+)-configuration at the α-carbon of glutamate was not so stringent since the D(−)-glutamate analogue was one-half as inhibitory as tetrahydrohomofolate. However, specificity was still observed in this part of the molecule, since the presence of an α-methyl substituent caused a marked decrease of inhibitory properties.

The α-amino-α,ω-dicarboxylate analogues from L(+)-aspartate to DL-α-aminopimelate showed good inhibition of the enzymatic reaction. Tetrahydrohomopteroyl aminopimelate was actually a better inhibitor than tetrahydrohomofolate.

**Table V**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Tetrahydropteroyl-L(+)-glutamate</td>
<td>0.120</td>
<td>0.9α</td>
</tr>
<tr>
<td>DL-Tetrahydropteroyl-DL-β-methylglutamate</td>
<td>0.060</td>
<td>1.6α</td>
</tr>
<tr>
<td>DL-Tetrahydropteroyl-DL-γ-hydroxyglutamate</td>
<td>0.015</td>
<td>0.9β</td>
</tr>
<tr>
<td>DL-Tetrahydropteroyl-L(+)-lysine</td>
<td>0.063</td>
<td>1.5α</td>
</tr>
</tbody>
</table>

* Based on only one active isomer.
* Based on only two active isomers out of the possible eight. See text for justification of this calculation.

The introduction of hydrophobic methyl groups on the β or γ carbons of glutamate caused little change in inhibition, whereas a substantial decrease in cofactor activity was observed with the corresponding tetrahydropteroylates (Table IV). The introduction of hydrophilic amino or hydroxyl groups on the γ carbons of glutamate resulted in a marked decrease in inhibition as well as in cofactor activity in the related tetrahydropteroylates.

The N°-tetrahydrohomopteroyllysine analogue was only about 3% as good an inhibitor as tetrahydrohomofolate. This
was surprising, since N*-tetrahydropteroylllysine was one of the better cofactors for this same enzyme.

The analogues of tetrahydrofolate that showed no cofactor activity with thymidylate synthetase were tested as inhibitors of the enzymatic reaction and were found to be inactive. Thus the tetrahydropteroyl compounds containing glycine, β-alanine, γ-aminobutyrate, DL-alanine, D(--)-glutamate, DL-α-methylglutamate, and DL-DL-γ-aminoglutamate have very little affinity for the enzyme at this level. This same lack of affinity was also observed for tetrahydropterate and tetrahydrohomopterate.

**DISCUSSION**

**General Comments on Dihydrofolate Reductase**—Dihydrofolate reductase catalyzes the reduction of folate to tetrahydrofolate as well as of 7,8-dihydrofolate to tetrahydrofolate. The latter reduction is by far the faster, and, since pteridines first emerge by synthesis de novo at the 7,8-dihydro level (16), it is this reduction that is of primary interest in biological systems. Studies of substrate specificity for this enzyme have been carried out in several laboratories (17, 18). It is clear that the 2-amino-4-hydroxy-6-substituted pteridine structure is required for substrate activity, although compounds without the p-aminobenzoylglutamate side chain may also be reduced (for example, 6-methylpteridinyl, biopertinyl, and 6-formylpyperidyl). A comprehensive review of the properties of dihydrofolate reductase is contained in the excellent doctoral thesis of Perkins (19).

The 2,4-diamino-6-substituted pteridine analogues of folate are inhibitors of the reduction of 7,8-dihydrofolate. Zakrzewski (20) has shown that the 2,4-diamino pteridine structure is sufficient to give inhibition of the enzymatic reaction. More recently, Baker (21) has shown that the 2,4-diamino pyrimidines carrying suitable side chains also inhibit the enzyme; however, these compounds are 1000-fold less inhibitory than aminopterin or amethopterin.

The only 2,4-dihydroxy-compound studied, 2-deamino-2-hydroxydihydrofolate (Table II), was neither a substrate of the enzyme nor an inhibitor of the reduction of dihydrofolate. Bertino (22) found that the unreduced compound was a relatively poor substrate for the same enzyme.

The interesting rate excitation shown by the halogenated dihydrofolates (Table I) cannot be attributed solely to the presence of a dipole near the site of reduction. Nearly the same rate enhancement was observed in the presence of 0.6 M sodium chloride solution (high dielectric constant) or p-mercuribenzoate (possible change in enzyme conformation) (23-25). Noteworthy also is the rate depression caused by the nitrosoamine structure at N€ (or N11 in dihydrohomofolate), which also introduces a dipole near the site of reduction. Since the nitroso group frequently mimics the carbonyl group in its chemical properties, it would have been expected that the N€-nitroso compounds would show behavior similar to N10-formyl dihydrofolate. The latter compound, however, is not a substrate of the enzyme at all; in fact, it is a good inhibitor of dihydrofolate reductase (17, 22).

Systematic variations in the glutamate area of folic acid recorded in Table III result in a 7-fold difference in reduction rate from the poorest substrate dihydropteroate, to the best substrate, dihydropteroylaminopimelate. The major favorable substrate property appears to be hydrophobic character in the vicinity of the γ-carboxyl of glutamate. One inference that may be drawn from Table III as well as from the work of other investigators is that the part of the enzyme with which glutamate interacts is distant from the active center actually involved in hydrogen transfer. Despite a fairly considerable accumulation of kinetic data obtained with dihydrofolate reductase in the presence of inorganic salts, mercurials, and denaturing agents (26), a meaningful rationalization of its behavior toward its many substrates is not yet possible.

**New Cofactors of Thymidylate Synthetase**—In this paper, synthetic analogues of tetrahydrofolate showing cofactor activity in thymidylate synthesis are described for the first time. Aminoadipate, aminopimelate, β-methylglutamate, and lysine analogues showed appreciable activity, although many other amino acid derivatives were ineffective. The specificity requirements of the enzyme for the amino acid region of tetrahydrofolate are a free α-carboxyl of glutamate and the L(+) configuration at the α-carbon of glutamate. The enzyme is less specific toward the γ position. A change in electrostatic charge or even loss of the charge was tolerated.

**New Inhibitors of Thymidylate Synthetase**—The inhibition of thymidylate synthesis by analogues of tetrahydrohomofolate paralleled the cofactor activity shown by analogues of tetrahydrofolate. A given substitution that reduced cofactor activity in a pteroyl derivative also gave reduction (although less marked) in the inhibitory activity of the analogous homopieroyl compound, indicating that those factors involved in the affinity of the tetrahydrohomofolates for the enzyme are also involved in the cofactor activities of the corresponding tetrahydrofolates.

Compounds containing the lysine skeleton were an exception to the above generalization. N*-Tetrahydropteroyl-L(+-)lysine and the corresponding compound containing the N*- (t-butyloxy carbonyl) group were among the better cofactors for thymidylate synthetase. The analogous tetrahydrohomopteroyl compounds, however, were very poor inhibitors of the enzymatic synthesis of thymidylate.

The relatively good inhibition of thymidylate synthetase by
the analogue of tetrahydrohomofolate containing d(-)-glutamate was unexpected. This was the first indication that inhibition in the homopteroyl series of compounds was not absolutely dependent on the nature or configuration of the amino acid. Undoubtedly, the different geometry of the 5,11-methylene-bridged compound compared to the 5,10-methylene-bridged compound (see Dreding stereomodels in Figs. 3 and 4) is directly responsible for a greater affinity with concomitant loss of cofactor activity, i.e., the inability of the enzyme to transfer the 1-carbon unit from tetrahydrohomofolate to deoxyuridine 5'-phosphate. It should be stressed that none of the tetrahydrohomofolate group of compounds has cofactor activity. The orientation of the tetrahydropteridyl and p-aminobenzoyl parts of the molecule is markedly different in N5,N10-methylene tetrahydrofolate and in N5,N11-methylene tetrahydrohomofolate. It is possible that the inability of N5,N10-methylene tetrahydrohomofolate to act as a cofactor in thymidylate synthesis is due to the higher position of the 1-carbon unit relative to the line between N5 and N10 (or N11), and to the more "open" arrangement of the tetrahydropteridyl and p-aminobenzoyl groups.

A comparison of properties of tetrahydrofolates containing one, two, and three methylene groups between position 6 of pteridine and the nitrogen of p-aminobenzoic acid is shown in Table VII. The three compounds are readily reduced by dithionite to 7,8-dihydro-derivatives. The dihydro-compounds are reduced by dihydrofolate reductase at essentially equal rates. Two of the tetrahydro-compounds show spectral shifts in the presence of formaldehyde. These are the compounds (tetrahydrofolate and tetrahydrohomofolate) that can form bridged compounds containing stable five- and six-membered rings. The third compound, tetrahydro-bis-homofolate, which would be expected to take these differences into account. Assuming pyramidal structures for N5 and N10, the hydrogen at carbon 6 is, in all conformations, on the opposite side of the tetrahydropyrazine ring from the 1-carbon unit which is to become the methyl group of thymidylate. The pyramidal structures are preferred for these nitrogens in analogy to similar molecules on which physical measurements have been made. N10 may be considered as analogous to alkyl anilines. The pyramidal structure for the nitrogen of such compounds is well established (27, 28). N5 and N10 are comparable to the amino groups of diamino pyrimidines. The pyramidal assignment in this case is derived from the x-ray diffraction study of Silverman and Yannoni (29) on a substituted diamino pyrimidine.

In analogy to chemical trans elimination reactions, it is tempting to speculate that the hydrogen at carbon 6 turns up in thymidylate, although clear-cut experimental evidence is not yet available. The model shown in Fig. 4 for 5,11-methylene tetrahydrohomofolate is based on the configuration for 5,10-methylene tetrahydrofolate. For the tetrahydrohomofolates, however, one other configuration is possible in which the hydrogen at carbon 6 is on the same side of the molecule as is the 1-carbon unit. Whether or not such a configuration is relevant to the inhibitory activity of tetrahydrohomofolate requires further study. Although insufficient data are available at the present time to allow speculation on the detailed mechanism of thymidylate synthesis, it should be noted that any future rationale will have to take these differences into account.

### Table VII

<table>
<thead>
<tr>
<th>Property</th>
<th>Tetrahydrofolate</th>
<th>Tetrahydrohomofolate</th>
<th>Tetrahydro-bis-homofolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stabilization by formaldehyde</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Spectral shift with formaldehyde</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ring stability</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cofactor for thymidylate synthetase</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Inhibitor of thymidylate synthetase</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Begins to show inhibition of thymidylate synthetase when the concentration is increased 100-fold over that of tetrahydrohomofolate.

A similar configuration for 5,10-methylene tetrahydrofolate requires some deformation of the Dreding stereomodels.

### REFERENCES


New Analogues of Folic Acid and Homofolic Acid

Enzyme Studies with New Analogues of Folic Acid and Homofolic Acid
Laurence T. Plante, Elizabeth J. Crawford and Morris Friedkin

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