ABSTRACT

The effects of the lipid-soluble dihydrofolate reductase inhibitor, trimetrexate, on the inhibition of thymidylate biosynthesis as a result of perturbation in cellular folate pools in H35 hepatoma cells in vitro has been investigated. Exposure of the cultures to increasing concentrations of trimetrexate between 2 and 20 nM causes a marked reduction in de novo thymidylate biosynthesis and a concomitant decrease in (6R,10S)-5,10-methylenetetrahydropteroylpolyglutamate (5,10-CH2H4PteGlu5) from 2.0–0.2 μM, respectively. This is accompanied by an increase in H2PteGlu, from 1.2 μM in control cultures to 4.7 μM in cultures exposed to 20 nM trimetrexate. The dependency of de novo thymidylate biosynthesis on intracellular 5,10-CH2H4PteGlu5, in trimetrexate-treated cells is compared with (a) the relationship of thymidylate biosynthesis on intracellular levels of 5,10-CH2H4PteGlu5 in folate-depleted cells supplemented with increments of folic acid and (b) the substrate (5,10-CH2H4PteGlu5) dependence of purified thymidylate synthase from the same source. All three results are nearly identical demonstrating that trimetrexate-dependent inhibition of de novo thymidylate biosynthesis is primarily a result of substrate depletion. These results coupled with the weak inhibitory properties of H2PteGlu5 for thymidylate synthase (Ki = 5.0 μM) suggest that H2PteGlu5 accumulation is not the major determinant in inhibiting thymidylate synthase following trimetrexate inhibition but under certain conditions has the potential to enhance the inhibition caused by substrate depletion.

INTRODUCTION

Many folate analogues have been developed for use in the chemotheraphy of neoplastic diseases (1–3) and a number of disorders related to the immune and inflammatory systems (4, 5). Most of the compounds that have been developed are tight binding inhibitors of DHFR2(1.5.1.3) (6–8), although more recent analogues have thymidylate synthase (EC 2.1.1.45) (9, 10) or GAR transformylase (EC 2.1.21) (11) as their primary enzyme target (Fig. 1). In recent years several laboratories have investigated the mechanism of inhibition caused by antifolates that block DHFR. Most of these studies have been directed at MTX, which is the most widely studied antifolate. The results of these investigations led to several postulates concerning the site and mechanism of action of MTX. It was originally felt that MTX caused a depletion of reduced folate coenzymes, accompanied by an accumulation of H2PteGlu5, due to the inhibition of DHFR (6). Thus, insufficient levels of 10-HCHOH2PteGlu5 and 5,10-CH2H4PteGlu5 would be present to support purine and thymidylate biosynthesis. More recently other mechanistic possibilities have been proposed, which include the inhibition of thymidylate synthase or 5-aminoimidazole-4-carboxamide ribonucleotide transformylase by H2PteGlu5, that accumulate during MTX blockade (12, 13). Alternatively, and perhaps additionally, the direct inhibition of thymidylate synthase (14) or possibly 5-aminoimidazole-4-carboxamide ribonucleotide transformylase by MTX polyglutamates has been suggested (15). These proposals are based upon earlier studies demonstrating the direct inhibition of thymidylate synthase by the polyglutamate derivatives of folates and antifolates (16–18).

It is known that DHFR inhibitors can inhibit both purine and thymidylate biosynthesis and that the relative sensitivity of each pathway varies among different cell types (1–3). Because of the complexity of these pathways it is possible that the mechanisms of inhibition of purine and thymidylate biosynthesis could differ. Thus, it is conceivable that thymidylate synthesis could be inhibited by folic acid depletion, whereas purine synthesis may be blocked by elevation of an inhibitory folate coenzyme or vice versa. To address the former question we evaluated the mechanism by which inhibition of DHFR causes a cessation in thymidylate biosynthesis. We have chosen trimetrexate as an inhibitor because of its inability to form polyglutamates and hence not directly inhibit thymidylate synthase (19, 20). Thus, the question that is being asked here is whether inhibitory levels of trimetrexate cause a sufficient reduction in 5,10-CH2H4PteGlu5 to stop thymidylate biosynthesis or is H2PteGlu5 accumulation and blockage of thymidylate synthase the predominant mechanism? To answer this question we have utilized enzymological analysis of thymidylate synthase isolated from H35 hepatoma cells and cell culture experiments.

MATERIALS AND METHODS

Materials. Swims S-77 medium, fetal calf serum, and horse serum were obtained from Grand Island Biological Co. (Grand Island, NY). Folic acid was purchased from Sigma Chemical Co. (St. Louis, MO) and purified by DEAE-cellulose (Schleicher & Schuell, Keene, NH) column chromatography prior to use (21). Trimetrexate was kindly provided by Drs. D. Fry and R. C. Jackson (Warner Lambert Laboratories, Ann Arbor, MI). [3H,5,7,13C]Folic acid, [6-H]deoxyuridine (26.7 Ci/mmol), [6-H]FdUMP (18 Ci/mmol) were purchased from Moravek Biochemical Inc. (Brea, CA), Sephadex G-25 and molecular weight standards were obtained from Pharmacia Fine Chemical Co. (Piscataway, NJ), and Ready/Solv was from Beckman (Fullerton, CA). SDS and all reagents for electrophoreses were provided by Bio-Rad Laboratories (Richmond, CA). PM10 ultrafiltration membranes were purchased from Amicon (Danvers, MA). PteGlu5 (n = 3, 5, and 7) were provided from Dr. B. Schircks Laboratories (Jona, Switzerland), and the samples were not less than 98% pure when evaluated by high performance liquid chromatography (22). All other chemicals were reagent grade.

Synthesis and Purification of H2PteGlu5 and 5,10-CH2H4PteGlu5. H2PteGlu5 was prepared according to the method of Blakley (23).
H₂PteGlu₂ were prepared in the same way except that, following titration to pH 2.8, the sample was placed in ice for 4 h to effect complete precipitation. This procedure gave approximately 75% yields of H₂PteGlu₂. The sample was placed in ice for 4 h to effect complete precipitation. H₂PteGlu₂ was prepared by enzymatic synthesis from H₃PteGlut using purified dihydrofolate reductase from *Lactobacillus casei* and glucose-6-dehydrogenase system for recycling NADPH (24). The reaction was performed at 37°C for 4 h in the dark under an argon atmosphere. H₃PteGlut was purified in the dark at 4°C by Sephadex G-25 gel filtration and DEAE-cellulose ion-exchange chromatography (0-2.0 M NaCl gradient) with 1% 2-mercaptoethanol (25). H₂PteGlu₂ was converted to 5,10-CH₂H₄PteGlu₂ by addition of 5.2 mM formaldehyde. (6R,5R)-10,5-CH₂H₄PteGlu₂ was not less than 95% pure by determining its concentration with thymidylate synthase and based upon the absorbance spectrum (26).

**Cell Culture.** H35 hepatoma cells free of *Mycoplasma* were grown in monolayer culture as described previously (21). All experiments were conducted following 72 h of culture unless otherwise indicated in the text. Cell growth and clonal assays were conducted as previously described (27). Folate-depleted H35 cells were obtained by placing cells that had been grown for 72 h under standard conditions (26) in folate-free medium and culturing for 96 h. These cultures were trypsinized and cultured in the same medium for 72 h with or without folate added and utilized experimentally at that time. In the absence of added folate, 5,10-CH₂H₄PteGlu₂ and H₂PteGlu₂ could not be detected in the cells by the assays described below. Thymidylate synthase-amplified H35 cells (H35F/F cells) were obtained by culturing H35 hepatoma cells in increasing concentrations of FdUrd starting with 0.3 mM in the presence of 25 μM folinic acid. After approximately 12 months the cells were able to grow in the presence of 100 nM of FdUrd. The cells from this FdUrd-resistant subline have been described before (28) and have an equivalent increase (approximately 60- to 70-fold) of thymidylate synthase when measured by enzyme assay (29) and by FdUMP binding (27).

**Thymidylate Biosynthesis.** Deoxyuridine incorporation was used to measure *de novo* thymidylate biosynthesis with cultured hepatoma cells. Following growth and trimetrexate exposure the cultures were exposed to 2 μCi [6-3H]FdUrd for 30 min at 37°C and incorporation was measured by a modification (27) of the method of Duch et al. (30). The results were expressed as dpm/mg cell protein. The incorporation of 2-14C-glycine (49.2 mCi/mmol, 2 mM) into DNA was used as a measure of *de novo* purine biosynthesis (31). Cell protein was measured by the method of Lowry et al. (32).

**Measurement of 5,10-CH₂H₄PteGlu₂, H₂PteGlu₂, and Glutamate Chain Length.** Cells were extracted with a solution of 50 mM Tris-1 mM EDTA-50 mM ascorbate (pH 7.4), boiled for 3 min, and centrifuged. To measure the amount of 5,10-CH₂H₄PteGlu₂, a sample containing 1-5 pmol of the folate was incubated with 20 pmol [3H]FdUMP (18 Ci/mmol) and 32 pmol of *L. casei* thymidylate synthase in a 200-μl volume at 25°C for 45 min. The reaction was stopped by adding 1% SDS and boiling for 8 min. The [3H]FdUMP-thymidylate synthase-10,CH₂H₄PteGlu₂ complex was quantitated following separation of unreacted [3H]FdUMP by Sephadex G-25 gel filtration (33). H₂PteGlu₂ was measured after being reduced to H₃PteGlut by *L. casei* DHFR in the presence of NADPH and then assayed as 5,10-CH₂H₄PteGlu₂ in the presence of 6.5 mM formaldehyde by the method described above. The amount of H₃PteGlut was calculated by subtracting the amount of 5,10-CH₂H₄PteGlu₂ present in the sample from the amount of 5,10-CH₂H₄PteGlu₂ present after *L. casei* DHFR treatment. Standard curves were generated using 5,10-CH₂H₄PteGlu₂ and H₂PteGlu₂ in extracts of folate-depleted cells. As a control to verify that H₂PteGlu₂ was being measured, MTX was added to the reaction, which resulted in a complete lack of detection of the added dihydro compound. The recovery of 5,10-CH₂H₄PteGlu₂ and H₂PteGlu₂ (after exposure to NADPH and DHFR) in the presence of excess formaldehyde is quantitatively complete following boiling for 3 min in 50 mM Tris-1 mM EDTA-50 mM ascorbate. The effect of boiling on 5,10-CH₂H₄PteGlu₂ in the absence of excess formaldehyde was examined by removing the formaldehyde by high performance liquid chromatography on a SAX column (Partisil Whatman, Clifton, NJ) AX 10 μ, 4.6 x 25 cm, 0.02-0.5 M sodium phosphate (pH 6.8) for 30 min at 1 ml/min on which the retention time for formaldehyde was 2-3 min and for 5,10-CH₂H₄PteGlu₂ was 23 min. Boiling a sample of the 5,10-CH₂H₄PteGlu₂ isolated by this procedure caused a 20% breakdown to H₂PteGlu₂ and the results of this study are corrected for that loss. Thiols were excluded from the extraction and boiling process since 0.4 μg 2-mercaptopethanol caused an extensive (80% under these conditions) conversion of 5,10-CH₂H₄PteGlu₂ to H₂PteGlu₂, a result which is consistent with previous studies (34, 35). The intracellular concentration of folates is calculated by dividing the total cell protein in mg by 3.15 which yields the total intracellular water volume (21). The polyglutamate chain length of CH₂H₄PteGlu₂ in cells was measured by electrophoresis of the unadenated ternary complexes (36). The current procedure differed in that thymidylate synthase and [3H]FdUMP was added to the buffer (50 mM Tris-HCl-1 mM EDTA-0.25 mM sucrose, pH 7.4) prior to cell extraction. In addition, 15 μl (15,000 dpm) or less was applied to electrophoresis. In the absence of these two modifications the primary chain length product in H35 cells could appear in the region of Glu₂ and Glu₄ (27), which is an underestimate of the actual chain length in H35 cells by one or two glutamates.

**Assay of Thymidylate Synthase.** Thymidylate synthase activity was determined by the spectrophotometric assay, which is based upon the change in absorbance (ΔA₃₄₀nm = 6.4 x 10⁻³) due to the conversion of 5,10-CH₂H₄PteGlu₂ to H₂PteGlu₂ (37). The enzyme activity during the column purification of the enzyme was monitored by tritium release from [5-3H]deoxyuridylate assay according to the method of Roberts (29). Protein concentration was determined by the dye-binding procedure of Bearden (38) using bovine albumin as a standard. Purification of Thymidylate Synthase from H35F/F Cells. Mass culture of H35F/F cells was conducted in 2× roller bottles (200 ml) (In Vitro Scientific Products) for 1 week in the absence of FdUrd and folinic acid. The cells were detached with trypsin, harvested, washed twice with phosphate-buffered saline, and stored as a pellet at -70°C. The cell pellets were thawed with 20 vols of buffer A (10 mM phosphate buffer, pH 7.4, 20 mM-2-mercaptoethanol, 0.005 mM dUMP), sonicated 3 min using Bronwill “Biosonik” in an ice-ethanol bath, and then centrifuged at 20,000 x g for 20 min at 4°C (fraction I, Table 1). The supernatant was treated with 0.1% proteamine sulfate and then centrifuged for 10 min (fraction II) followed by treatment with 75% ammonium sulfate and the same centrifugation to precipitate the enzyme (fraction III). The precipitate was dissolved in several volumes of buffer A containing 0.1% Triton X-100 (buffer B) and passed slowly through a 10-formyl-5,8-dideazafolate ethyl-Sepharose 4B column (1 x 3.5 cm) (39) saturated previously with buffer B. The column was washed with 100 ml of buffer B and then with 100 ml of 0.5 mM phosphate buffer, pH 7.4, containing 1 mM MgCl₂, 20 mM-2-mercaptoethanol, 0.005 mM dUMP, and 1% Triton X-100. The
TRIMETREXATE INHIBITION OF THYMIDYLATE BIOSYNTHESIS

Table 1 Purification of thymidylate synthase from H35 F/F cells

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>14.8</td>
<td>390</td>
<td>0.038</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(I) 0.1% potaminesulfate</td>
<td>11.6</td>
<td>205</td>
<td>0.056</td>
<td>78</td>
<td>1.4</td>
</tr>
<tr>
<td>(II) 0-75% (NH₄)₂SO₄</td>
<td>10.0</td>
<td>124</td>
<td>0.082</td>
<td>67</td>
<td>2.1</td>
</tr>
<tr>
<td>(III) Affinity column (IV)</td>
<td>6.5</td>
<td>12.7</td>
<td>0.51</td>
<td>43</td>
<td>13.2</td>
</tr>
<tr>
<td>(V) DEAE-cellulose column (VI)</td>
<td>5.6</td>
<td>10.0</td>
<td>0.56</td>
<td>38</td>
<td>14.7</td>
</tr>
</tbody>
</table>

PHOSPHORYLASE B 94,000
BOVINE SERUM ALBUMIN 67,000
OVALBUMIN 43,000
CARBONIC ANHYDRASE 30,000
TRYPsin INHIBITOR 20,100
LACTALBUMIN 14,400

Fig. 2. SDS-polyacrylamide gel electrophoresis at different stages of the purification of thymidylate synthase from H35F/F cells. Std, standard; roman numerals, steps in the purification described in Table 1; numbers on ordinate, molecular weight.

enzyme was eluted with 400 ml of 0.1 M phosphate buffer, pH 7.4, containing 0.5-1 M KCl, 1 mM MgCl₂, 20 mM 2-mercaptoethanol, and 0.1% Triton X-100 (fraction IV). Fractions with thymidylate synthase activity were pooled and concentrated by ultrafiltration (PM10 membrane; Amicon, Danvers, MA), dialyzed overnight against buffer B, and passed through a DEAE-cellulose column (1 x 5 cm) equilibrated previously with buffer B. The enzyme was eluted with 0.2 M phosphate buffer containing 1 mM MgCl₂, 20 mM 2-mercaptoethanol, 0.1% Triton X-100, and 20% sucrose (fraction V). The last step of purification removed trace contaminants (<10%). The purity of the final product is shown in Fig. 2 which was analyzed by polyacrylamide gel electrophoresis (40) using the Mini-Protean electrophoresis system (Bio-Rad Laboratories, Richmond, CA) (Fig. 2).

RESULTS AND DISCUSSION

Effect of Trimetrexate on de Novo Thymidylate Biosynthesis. The capacity of trimetrexate to inhibit deoxyuridine incorporation in dividing H35 cells is shown in Fig. 3. Little effect occurs when the cells are incubated in the presence of 2 nM trimetrexate. Extensive inhibition is noted when trimetrexate is increased to 5 nM (70%), while inhibition reaches 91% at 20 nM trimetrexate. Trimetrexate-dependent inhibition of de novo thymidylate synthesis by measuring the release of tritium from [5-3H]dUrd (39) or by inhibition of thymidine incorporation into DNA (42) show essentially identical results. Outgrowth studies and clonal assay response to trimetrexate showed a response similar to inhibition of deoxyuridine incorporation. Inhibition of de novo purine biosynthesis was less sensitive to trimetrexate, with significant inhibition occurring in the range of 15-20 nM. This extensive discrepancy in sensitivity of purine and pyrimidine biosynthesis to DHFR inhibition is not observed when MTX is used as a DHFR inhibitor.

Effect of Trimetrexate on Cellular 5,10-CH₂H₄PteGlu₉ and H₂PteGlu. The effect of inhibitory concentrations of trimetrexate on the cellular concentrations of the folate substrate (5,10-CH₂H₄PteGlu₉) and product (H₂PteGlu) of the thymidylate synthase reaction was measured. The major polyglutamate form of both folates in control and trimetrexate-inhibited culture is the heptaglutamate (Fig. 4). When cells were exposed to 2 nM trimetrexate for 20 h, the cellular pool of 5,10-CH₂H₄PteGlu₉ was reduced approximately 33% with no reduction in deoxyuridylate incorporation (Table 2 and Fig. 3). Increasing the concentration of trimetrexate through its inhibitory range causes a marked reduction in 5,10-CH₂H₄PteGlu₉ with the greatest decrease in de novo thymidylate biosynthesis (Fig. 3) occurring as the 5,10-CH₂H₄PteGlu₉ concentration is reduced from 2.0-0.3 μM (Table 2). The effect of trimetrexate on H₂PteGlu shows little change when the inhibitor is present at 2 nM but increases at higher trimetrexate concentrations. The maximal intracellular concentration achieved in this study is 4.7 μM at 20 nM trimetrexate.

Concentration Dependence of Thymidylate Biosynthesis on Cellular 5,10-CH₂H₄PteGlu₉. To determine the concentration of 5,10-CH₂H₄PteGlu₉, required to maintain thymidylate biosynthesis, folate-depleted cells were treated with increments of

Fig. 3. The effect of trimetrexate on H35 cells. Cultures of H35 cells were exposed to the indicated concentration of trimetrexate and the effect on growth (•), colony-forming ability (•), de novo purine (D), and thymidylate biosynthesis (O) were measured as described in "Experimental Procedures." The cultures utilized for purine and thymidylate biosynthesis were exposed to trimetrexate only during the last 20 h of the 72-h growth cycle.

*M. S. Rhee and J. Galivan. unpublished observations.
and $H_2PteGlu^7$ were measured as nmol/g cell protein, which was converted to cell volume by factor 3.15.  Isolated to null volume also gave a value of Glu, for all experimental points.  Application of a series of dilutions of the sample extracts extrapolated to null volume also gave a value of Glu for all experimental points.

**Table 2 Trimetrexate concentration response on cellular 5,10-CH$_2$H$_4$PteGlu, and H$_2$PteGlu, concentration**

<table>
<thead>
<tr>
<th>Trimetrexate (nM)</th>
<th>5,10-CH$_2$H$_4$PteGlu $^a$ (µM)</th>
<th>H$_2$PteGlu $^a$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.0 ± 0.47 $^b$</td>
<td>1.2 ± 0.44</td>
</tr>
<tr>
<td>5</td>
<td>2.0 ± 0.55</td>
<td>1.8 ± 0.11</td>
</tr>
<tr>
<td>10</td>
<td>0.6 ± 0.21</td>
<td>3.2 ± 1.14</td>
</tr>
<tr>
<td>15</td>
<td>0.3 ± 0.13</td>
<td>3.6 ± 0.27</td>
</tr>
<tr>
<td>20</td>
<td>0.22 ± 0.08</td>
<td>4.4 ± 0.21</td>
</tr>
<tr>
<td>0.15 ± 0.05</td>
<td>4.7 ± 0.30</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SD (n = 5).

**Table 3 Effect of cellular 5,10-CH$_2$H$_4$PteGlu, concentration on de novo thymidylate synthase in folate-depleted H35 cell**

<table>
<thead>
<tr>
<th>Folic Acid (µM)</th>
<th>5,10-CH$_2$H$_4$PteGlu $^a$ (µM)</th>
<th>H$_2$PteGlu $^a$ (µM)</th>
<th>6-[H]dUrd (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.10</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.25</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.34</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.57</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>0.40</td>
<td>0.71</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>0.60</td>
<td>1.2</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>1.73</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>4.00</td>
<td>2.2</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

* Average of 2 experiments.

We also studied the inhibitory effect of H$_2$PteGlu, on the purified thymidylate synthase against 5,10-CH$_2$H$_4$PteGlu, of equivalent glutamate chain length. The $K_v$ were 6.2, 5.5, and 5.0 µM for the mono-, penta-, and heptaglutamates, respectively.  H$_2$PteGlu, was found to be a noncompetitive inhibitor of the enzyme [by using Dixon plot analysis (43)] regardless of the glutamate chain length.  The methotrexate polyglutamate 4NH$_2$-10-CH$_2$PteGlu, [the major cellular polyglutamate form of methotrexate in this cell line (44)] is also a noncompetitive inhibitor that is 100-fold more inhibitory than the polyglutamate derivatives of H$_2$PteGlu.  The similarity of the amplified thymidylate synthase to the enzyme from wild-type H35 cells was examined by kinetic analysis.  Wild-type enzyme was purified to step III and evaluated for the $K_v$ for 5,10-CH$_2$H$_4$PteGlu, and Glu, and dUMP and the $K_v$ for H$_2$PteGlu $^a$. The results were indistinguishable from those for the enzyme from H35 F/F cells.  The kinetic values show a high degree of qualitative and quantitative similarity to the thymidylate synthase that has been studied when isolated from MCF-7 breast cancer cells (13, 14).

A compilation of the results from Tables 2-4 can be devised by plotting thymidylate biosynthesis as a function of 5,10-CH$_2$H$_4$PteGlu, concentration with (a) isolated thymidylate synthase, (b) H35 cells blocked in folate coenzyme function with trimetrexate, and (c) folate-depleted cells which have been restored with added folic acid (Fig. 5).  Trimetrexate is useful in this study as the DHFR inhibitor because it cannot be converted to a direct inhibitor of thymidylate synthase and its inhibitory effect at these concentrations impeded de novo thymidylate biosynthesis selectively, i.e., without inhibiting purine biosynthesis at low concentrations (Fig. 3).

The dependence of thymidylate biosynthesis on 5,10-CH$_2$H$_4$PteGlu, concentration with isolated thymidylate synthase, folate-depleted cells, and trimetrexate-impaired cells shows a strong correlation.  The requirement for 5,10-CH$_2$H$_4$PteGlu, for thymidylate biosynthesis is nearly identical when the requirement of the isolated enzyme is compared with that of folate-depleted cells.  The analogous curve for the trimetrexate-treated cells is similar, requiring only slightly more 5,10-CH$_2$H$_4$PteGlu, (the major cellular form) for one-half saturation of the reaction.  The requirement of a higher concentration (approximately 0.2 µM) of 5,10-CH$_2$H$_4$PteGlu, to support thymidylate biosynthesis in the trimetrexate-inhibited cells may be related to H$_2$PteGlu.  An analysis of the enzymatic results (Table 3) show that at 0.6 µM, 5,10-CH$_2$H$_4$PteGlu, the rate of thymidylate synthesis with the isolated enzyme is 50% of that
at substrate saturation and this is reduced to 30% with the inclusion of 3.0 μM H₂PteGlu₄. These results agree with results in Table 2 and Fig. 3 where 5 nM trimetrexate causes a reduction in 5,10-CH₂H₄PteGlu₁ to 0.6 μM and an increase in H₂PteGlu₄ to 3.2 μM, resulting in a reduction in de novo thymidylate biosynthesis to 30% of control. Thus, the intact cell data correlate with an analogous experiment using the isolated enzyme. The amount of inhibition of isolated thymidylate synthase with 4.7 μM H₂PteGlu₂ (the maximal amount observed in trimetrexate-inhibited cells) at all substrate concentrations tested is 46 ± 1% (n = 4) which is inadequate to account for a mechanism in which trimetrexate causes a blockage of thymidylate synthase solely as a result of elevated H₂PteGlu₄. In contrast, it would appear that the reduction in 5,10-CH₂H₂PteGlu, that occurs following trimetrexate inhibition is sufficient to cause at least a 90% inhibition in thymidylate biosynthesis (Fig. 3; Table 2). Thus, the chief determinant of the loss of thymidylate biosynthesis in hepatoma cells treated with trimetrexate appears to be a reduction in 5,10-CH₂H₂PteGlu₄.

Based upon enzymological studies in several laboratories (14-17, 45, 46) and the enhancement of H₂PteGlu₄ in cells following DHFR inhibition (14, 47), it has been postulated that the escalation in H₂PteGlu₄ concentration and resultant inhibition of thymidylate synthase play a role in the inhibitory activity of folate analogs which block DHFR (13). In the present study, H₂PteGlu₄ is clearly not the major cause of this inhibition which appears to be more closely related to the loss in substrate. Although this hypothesis has not been widely tested with regard to trimetrexate, recent studies with L1210 cells by Goldman et al. (47, 48) have evaluated the effects of antifolates on the radiolabeled precursor incorporation into nucleotides, de novo thymidylate biosynthesis, and folate dynamics by experimental and computer-modeling studies. Analysis of their results and a comparison to the activity of MTX in that study resulted in the conclusion that trimetrexate was reducing thymidylate synthase by depleting cellular reduced folates and not by the inhibitory effects of H₂PteGlu₄. Those studies along with the direct measurements of the relationship of thymidylate biosynthesis to 5,10-CH₂H₂PteGlu₄ concentration made here demonstrate that H₂PteGlu₄ is not the major determinant in inhibiting thymidylate synthase in these cell systems when trimetrexate is used as a DHFR inhibitor. However, it may intensify the inhibition of thymidylate synthase, particularly at very low and high concentrations of 5,10-CH₂H₂PteGlu₄ and H₂PteGlu₄, respectively. If the cells were to generate dramatically higher levels of H₂PteGlu₄, it could have more significant effects, although the amounts of H₂PteGlu₄ following DHFR blockage remain in the present range or less in this system and others (15, 47-50).

It should be noted that the effect of MTX in this system might be vastly different than trimetrexate due to the potent capacity of the polyglutamates to directly inhibit thymidylate synthase (Table 4) (14-17, 47-50). This is particularly true with regard to H35 cells in which as much as 100 μM MTX-polyglutamates can be generated. Studies analogous to the present one utilizing MTX and 4-fluoro-MTX (51) are underway to examine that point.

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Role of Substrate Depletion in the Inhibition of Thymidylate Biosynthesis by the Dihydrofolate Reductase Inhibitor Trimetrexate in Cultured Hepatoma Cells

Myung S. Rhee, Malgorzata Balinska, Marlene Bunni, et al.