Intracellular Metabolism of 5-Formyl Tetrahydrofolate in Human Breast and Colon Cell Lines

Donna M. Boarman and Carmen J. Allegra

Medicine Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20892

ABSTRACT

This report describes the intracellular metabolism of 5-formyltetrahydrofolate into the various one-carbon substituted folate and polyglutamate pools in a human breast (MCF-7) and colon (HCT 116) carcinoma cell line. Metabolism into the one-carbon substituted pools was found to be time and dose dependent over a concentration range up to 50 μM. A 3-fold increase in total intracellular folate was noted over a 50-fold concentration range (1–50 μM) of 5-formyltetrahydrofolate tested in the colon cell line, while in the breast line, a 6-fold increase was detected over a 500-fold concentration range (0.1–50 μM). The level of 5, 10-methylenetetrahydrofolate, which was detectable only in the breast cell line, was found to increase by a factor of 10 (1.8 pmol/mg to 17.9 pmol/mg) over the concentration range studied. The majority of metabolism was into the 10-formyltetrahydrofolate and tetrahydrofolate pools in the breast cells and into the 5-methyltetrahydrofolate pool in the colon cells. Polyglutamation was also time and dose dependent, with a significant proportion of the total pool represented by the higher polyglutamate forms (Glu3—Glu5) after 24 h of continuous exposure to 5-formyl tetrahydrofolate. Pentaglutamate was the highest level noted in both cell lines. The intracellular half-life of the polyglutamate forms was inversely related to the length of the polyglutamate tail with half-lives of 71, 131, 143, 441, and 1167 min for the mono- through pentaglutamate, respectively. Finally, up to a 20:1 ratio of the biologically inactive (6R) isomer to active (6S) isomer of 5-formyltetrahydrofolate resulted in no effect on metabolism into the one-carbon substituted folate pools and only minimal decreases in metabolism to the polyglutamate forms. These studies suggest that prolonged exposure to even relatively low doses of 5-formyltetrahydrofolate may be optimal for intracellular metabolism to the most biologically relevant forms for ternary complex formation with thymidylate synthase and fluorodeoxyuridylate, since longer exposures result in a greater accumulation of the higher polyglutamates.

INTRODUCTION

5-Formyl-H4PteGlu2 (Leucovorin, citrovorum factor) has become the standard of care when used in combination with 5-fluorouracil for the treatment of patients with advanced colon and rectal carcinoma. The biochemical basis for the activity of these agents when used in combination has been reviewed recently (1). In brief, 5-fluorouracil is metabolized in malignant and normal cells to several active anabolites including 5-fluorouridine monophosphate, which is a potent inhibitor of the enzyme thymidylate synthase. Inhibition occurs via a covalent complex with the enzyme in the presence of the folate substrate, 5, 10-methylene tetrahydrofolate. Since the stability of the ternary complex is dependent on the intracellular folate concentration, 5-formyl-H4PteGlu, a stable reduced folate, has been used to augment these pools. Early studies from several laboratories clearly demonstrated that the addition of 5-formyl-H4PteGlu to cells in culture could augment the cytotoxic effects of the fluoropyrimidines (2, 3). Since these initial reports, many investigators have found that optimization of the 5-fluorouracil activity occurs when cells are treated with 1–20 μM concentrations of 5-formyl-H4PteGlu (4–6). These preclinical observations have been extended to the treatment of patients with a variety of solid tumors including colorectal (7–9), breast (10, 11), gastric (12, 13), and head and neck carcinomas (14) with the combination of fluoropyrimidines and 5-formyl-H4PteGlu. These clinical studies have demonstrated that the addition of the folate to the fluoropyrimidines results in an enhanced response rate in patients with advanced colorectal carcinoma (7–9) and prolonged survival (7, 9) compared to therapy with fluoropyrimidines alone. Since a variety of doses and schedules of 5-formyl-H4PteGlu have been used clinically, one of the critical questions concerns the optimal dose and scheduling of the folate. Thus, far, it appears that in using the daily schedule for 5 days, the dose of 5-formyl-H4PteGlu over a 10-fold range (20 versus 200 mg/m2/d) results in a similar response and survival in patients with advanced colorectal carcinoma (7), while low doses (50 mg/m2) on the intermittent weekly schedule are inferior compared to the higher dose (500 mg/m2) regimen (8). Since 5,10-methylene-H4PteGlu represents only a minor fraction of the rapidly interconvertible intracellular folate pool in mammalian cells (5%) (15), it would be of interest to understand the metabolism of 5-formyl-H4PteGlu into all of the various one-carbon substituted states and polyglutamate forms. Polyglutamation of 5,10-methylene-H4PteGlu markedly enhances its affinity for thymidylate synthase (10-fold) and has been shown to be greater than 100-fold more potent as a potentiator of ternary complex formation with human enzyme when compared to the monoglutamate form (16, 17). Recent investigations have focused on the metabolism of 5-formyl-H4PteGlu to 5,10-methylene H4PteGlu, including its polyglutamate forms in a human colon xenograft model in immune-deprived mice (18–20). These investigators found that the combined pool of H4PteGlu and 5,10-methylene-H4PteGlu increased up to 4-fold following 24-h infusions of 5-formyl-H4PteGlu at a dose of 1000 mg/m2. Interestingly, despite the intracellular accumulation of polyglutamate forms following folate exposure, a rapid decline of the folates to baseline levels was noted to occur in concert with the decline in serum levels. A study by Romani et al. (21) examined folate metabolism in both a parent CCRF-CEM cell line and a subline (CCRF-CEM/P) which was deficient in its ability to synthesize polyglutamate forms due to an alteration of folylpolyglutamate synthetase (21). They observed a similar dose-dependent increase in the two cell lines in the 5,10-methylene-H4PteGlu pool after exposure to 5-formyl-H4PteGlu; however, they found decreased polyglutamation of this folate in the CCRF-CEM/P cell line when compared to the parental line. While these investigators found increased inhibition of thymidylate synthase in the wild-type cells with the addition of 5-formyl-H4PteGlu to 5-fluorouracil, there was no enhanced inhibition of thymidylate synthase in the CCRF-CEM/P cell line. These data support the importance of polyglutamation in the efficacy of...
the 5-formyl-H$_4$PteGlu-fluorouracil combination.

Since the various folate one-carbon substituted forms including their polyglutamate state may influence and contribute to the formation of ternary complex, we wished to investigate the metabolism of 5-formyl-H$_4$PteGlu to these states in intact human cells. In defining the intracellular metabolism of 5-formyl H$_4$PteGlu we hope to provide additional insight into the optimal clinical use of this agent. We chose a human colon (HCT 116) and breast (MCF-7) cell line as model systems since these two histologic types have been found to be clinically responsive to the use of 5-formyl-H$_4$PteGlu with 5-fluorouracil.

MATERIALS AND METHODS

(6S)-5-Formyl-[3',5',7'-3H]H$_4$PteGlu (specific activity, 40 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). The radiopurity of the compound determined by HPLC was ≥98% and was used without further purification. Minimal essential medium without folic acid was purchased from Gibco (Gaithersburg, MD). Glutamine, phosphate-buffered saline, and fetal calf serum were purchased from Biofluids (Rockville, MD). Sep-Pak C$_18$ cartridges and Pic Reagent A were purchased from Waters Associates (Milford, MA). (6R, S)-5-formylH$_4$PteGlu was obtained from Burroughs Wellcome Co (Research Triangle Park, NC); (6R)-5-formyl-H$_4$PteGlu and (6S)-5-formyl-H$_4$PteGlu were kindly provided by Lederle Laboratories (Pearl River, NY). β-Mercaptoethanol, albumin (fraction V), p-aminobenzoate-L-glutamic acid, and reduced folate standards including (6R, S)-tetrahydrofolate polyglutamate were purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). Pico Aqu Scintillation cocktail was purchased from Packard (Meriden, CT). PteGlu polyglutamate standards were purchased from Sigma Chemical Co. (St. Louis, MO). 5,10-Methylene tetrahydrofolate acid was prepared as described previously (19). Methanol was purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). Pico Aqua scintillation cocktail was purchased from Packard (Meriden, CT). PteGlu polyglutamate standards were purchased from Schircks Laboratories (Jona, Switzerland). Methotrexate polyglutamate standards were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). 5-Formyl-H$_4$PteGlu was a generous gift from Dr. Anthony Fitzhugh (Frederick, MD).

Cell Lines

An early-passage human MCF-7 breast cancer and a human HCT 116 colon cell line were used for these experiments. The characterization of these cells has been described previously (22, 23). The cells were grown as a continuous monolayer in 75-cm$^2$ plastic tissue culture flasks (Falcon Labware, Oxnard, CA) in minimal essential media without folic acid and supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 nM (6S)-5-formyl-H$_4$PteGlu. All cells were grown in folate-deplete media for at least two passages before use in the experiments. For each of the experimental points, 1 x 10$^6$ cells were plated onto 75-cm$^2$ plastic tissue culture flasks, and after 96 h of growth (60–70% confluency) the cells were used for the various experiments.

Intracellular Folate Pool Measurements

MCF-7 and HCT 116 cells were exposed to various concentrations of (6S)-5-formyl-[3H]H$_4$PteGlu ranging from 0.1 to 50 nM for 2, 6, and 24-h time intervals. The specific activity of the 5-formyl-[3H]H$_4$PteGlu was diluted by the addition of unlabeled (6R, S)-5-formyl-H$_4$PteGlu necessary to achieve the desired final concentrations, i.e., the concentrations listed in the figures and tables represent the final concentration of the racemic folate except where specifically indicated. At the end of the specified exposure time, the cells were washed two times with ice-cold phosphate-buffered saline and then harvested in 1 ml of the saline with the aid of a rubber cell scraper. A 100-μl aliquot was removed for subsequent protein analysis. The folates were extracted from the remainder of the cell suspension according to previously published techniques (24, 25). Briefly, the cells were lysed and folates extracted by boiling for 90 s in 2 ml of a 2% ascorbate, 2% β-mercaptoethanol solution, pH 6.0. The denatured protein was removed by centrifugation at 10,000 x g for 5 min. The supernatant was then treated with 24 units (1 unit = 1 nmol of product formed/min/mg) of partially purified hog kidney polyglutamate hydrolase (specific activity, 2 units/mg) at 37° for 30 min. The hydrolase was partially purified from fresh hog kidneys (10–13 mg protein/ml) according to published methods (26). The solution was then subjected to a 90-s boil after the addition of 2 ml of the 2% ascorbate, 2% β-mercaptoethanol solution and centrifuged at 10,000 x g for 5 min to remove the denatured proteins. The folates were then concentrated by extraction with a C$_18$ Sep-Pak cartridge, followed by evaporation under a steady stream of nitrogen.

The labeled folates were separated by HPLC using a Waters Model 510 pump and a Waters Model 440 UV absorption detector with a fixed wavelength of 256 nm according to methods described previously (24, 25). A 0.8 x 10 cm C$_18$ Bondapak column (particle size, 10 μm spherical) was developed using a flow rate of 2 ml/min under isocratic conditions. The mobile phase consisted of 80% Pic Reagent A (25:1 v/v, water:Pic A), adjusted to pH 5.5 with 1 N HCl and 20% methanol. The separated labeled folate pools were quantitated using an in-line liquid scintillation counter (Model Flo-One Beta, Radiomatic Instruments, Meriden, CT). The identity of the various physiological folates was authenticated by coelution with unlabeled standard folates injected with each assay sample. Folates were further identified by acting as substrates for specific enzymatic reactions as previously detailed (24, 25). The recovery rate for the various folates (except 5,10-methylene H$_4$PteGlu) ranged from 60 to 70%, and no correction for recovery was applied since all comparisons were made between cells processed under identical conditions and at the same time. The retention times for the folates were as follows: paraaminobenzoate, 4–5 min; paraaminobenzoate-glutamate, 7.5–8 min; 10-formyl-H$_4$PteGlu, 10 min; H$_4$PteGlu, 12.5 min; 5-formyl-H$_4$PteGlu, 15 min; H$_4$PteGlu, 18.5 min; and 5-methyl-H$_4$PteGlu, 26 min.

We also examined the fate of these intracellular folate pools at several time points after a 24-h exposure to 5-formyl-H$_4$PteGlu and after the 5-formyl-H$_4$PteGlu had been removed from the media. After exposure to 10 μM 5-formyl-H$_4$PteGlu for 24 h, the cells were washed two times with phosphate-buffered saline and fresh medium was added for an additional 2, 6, and 24 h of incubation. The cells were then harvested and the folates were extracted as described above.

5,10-Methylene-tetrahydrofolate Quantitation

HPLC Method. The 5,10-methylene-H$_4$PteGlu pool was quantitated in a separate set of experiments using cells labeled with (6S)-5-formyl-[3H]H$_4$PteGlu (15). For these experiments, the cells were exposed to concentrations of 5-formyl-H$_4$PteGlu ranging from 0.1 to 50 μM for 24 h. At the end of the 5-formyl-H$_4$PteGlu incubation, the cells were washed twice with ice-cold phosphate-buffered saline and harvested. The intracellular folates were extracted as described and separated by HPLC using a modified mobile phase consisting of 76% Pic A, adjusted to pH 4.0 with 1 N HCl, and 24% methanol. The folate pool was quantitated using an in-line scintillation counter. The retention time for 5,10-methylene-H$_4$PteGlu was 23 min. The recovery rate for this folate was 36 ± 8 (SD) % (15). Authentication of the 5,10-methylene-H$_4$PteGlu was by coelution with standard compound and by specific metabolism of the putative 5,10-methylene-H$_4$PteGlu peak to H$_4$PteGlu in the presence of thymidylate synthase and deoxyuridylate (15).

Enzyme Binding Methods. The 5,10-methylene-H$_4$PteGlu pools were also quantitated by a radioenzymatic binding assay. This method is a modification of the assay originally developed by Priest (27) and is based upon the binding of 5,10-methylene-H$_4$PteGlu to thymidylate synthase and [3H]FdUMP to form a stable ternary complex. The recovery rate for the folate using this assay was 78 ± 4% (15).

Intracellular Folate Polyglutamate Measurements

MCF-7 cells were exposed to 10 μM 5-formyl-[3H]H$_4$PteGlu concentrations for 2, 6, and 24 h. The HCT 116 cells were exposed to both 1 and 10 μM 5-formyl-[3H]H$_4$PteGlu for the same time intervals. At the
end of each time period the cells were washed two times with ice-cold phosphate-buffered saline and harvested in 1 ml of the saline with the aid of a rubber cell scraper. A 100-μl aliquot was removed for protein quantitations. The folate polyglutamates were extracted from the remainder of the cell suspension by boiling for 90 s in 2 ml of a 2% ascorbate, 2% mercaptoethanol solution, pH 6.0. The denatured protein was removed by centrifugation at 10,000 × g for 5 min. The polyglutamated forms were then concentrated by use of a C18 Sep-Pak cartridge and separated by HPLC using a 22-min linear gradient from 20% to 35% acetonitrile in Pic A (pH 5.5). The separated polyglutamates were quantitated by an in-line liquid scintillation counter. Under these separation conditions, we found that the polyglutamate tail length was the critical determinant of retention time. The various folate polyglutamates (Glu1—Glu5) extracted from the human breast cancer cells had a similar elution pattern compared with both folic acid polyglutamates (Glu1—Glu5) and pABA polyglutamates (Glu1—Glu5), which were cleaved from their respective folate polyglutamates (28) (Fig. 1, A—C). The dihydrofolate polyglutamates, generated as previously described (16), also coeluted with the respective polyglutamates of folic acid and pABA as illustrated in Fig. 1D. The retention times for these compounds are listed in Table 1. The retention times of the methotrexate polyglutamates are also shown in Table 1 in comparison to the folate polyglutamates. These polyglutamates differ by up to 3 min for the mono- and diglutamate forms when compared with the respective folate polyglutamates. Since the differences in retention times were greatest for the monoglutamates (as expected, given the least number and therefore the least contribution from the glutamate residues) and because of the availability of these forms, various other one-carbon substituted folate monoglutamates, including 5-formyl-H4PteGlu, 5-methyl-H4PteGlu, 5,10-methylene-H4PteGlu, and 10-formyl-H4PteGlu, were examined and are listed in Table 1. These folate monoglutamates had retention times within 1 min of each other. The diglutamates of both 5-formyl-H4PteGlu and 10-formyl-H4PteGlu had retention times indistinguishable from the other diglutamates tested. H4PteGlu5 and 5,10-methylene-H4PteGlu5 coeluted with PteGlu5, pABA5, and H2PteGlu5. These observations suggested that the folate polyglutamates profile could be accurately quantitated without the need for C9—10 bond disruption as previously described (28). The identities of the various polyglutamates were therefore authenticated by coelution with unlabeled PteGlu polyglutamate standards.

To measure the amount of polyglutamates which egressed from the cell over time, a separate set of experiments was performed in which the cells were first exposed to 5-formyl-[3H]H4PteGlu for 24 h. The breast cells were exposed to 1 μM and the colon cells to either 10 or 100 μM 5-formyl-H4PteGlu. At the end of 24 h the cells were washed twice with phosphate-buffered saline, and fresh medium was added for an additional 2-, 6-, and 24-h period. At the end of the specified time period, the cells were again washed two times with ice-cold phosphate-buffered saline, harvested in 1 ml of the same and quantitated according to the methods described above.

### Protein Measurement

A 100-μl aliquot of cell suspension was sonicated with five 3-s bursts using a Branson model 350 sonicator equipped with a microtip. The cell debris was pelleted by centrifugation at 10,000 × g for 10 min, and the protein in the supernatant was quantitated using the method of Bradford (29).

### Calculations

Disintegrations per minute, obtained by counting an aliquot of the extracted labeled folates, were converted to total intracellular folate content in pmol/mg protein by dividing total disintegrations/min/flask of cells by total protein/flask of cells and then dividing by the specific activity of the labeled 5-formyl-H4PteGlu: (disintegrations/min/flask)/(mg protein/flask)/(disintegrations/min/pmol of folate) = pmol of folate/mg protein.

### RESULTS

We examined the metabolism of 5-formyl-H4PteGlu during 24-h exposures in the human breast (MCF-7) cell line over a 500-fold range of 5-formyl-H4PteGlu concentration (0.1—50 μM) and in the human colon (HCT 116) cell line over a 50-fold range of 5-formyl-H4PteGlu concentration (1—50 μM). The composition of the folate pools in cells exposed to 1 μM 5-formyl-H4PteGlu differed between the two lines: 80% 5-methyl-H4PteGlu, 15% 10-formyl-H4PteGlu, and 5% H4PteGlu for the colon line; and 27% 5-methyl-H4PteGlu, 32% 10-formyl-H4PteGlu, 36% H4PteGlu, and 5% 5,10-methylene-H4PteGlu for the breast line. In both cell lines we found rapid metabolism of 5-formyl-H4PteGlu to the various folate forms, since less than 2% of the total intracellular labeled folates were detectable as 5-formyl-H4PteGlu even at the highest exposure dose of 50 μM (data not shown). Exposure to 50 μM 5-formyl-H4PteGlu resulted in a 3-fold expansion of the total folate pools for both the HCT 116 and MCF-7 cells when compared to those cells grown in 1 μM 5-formyl-H4PteGlu and in a 6-fold expansion for the breast cells compared with those grown in 0.1 μM 5-formyl-H4PteGlu. The increase in folate pools in the colon cells was primarily due to an expansion of the 5-methyl-H4PteGlu pool (Fig. 2), while in the breast cells (Fig. 2) this increase was

### Table 1 HPLC retention times of various polyglutamated compounds

<table>
<thead>
<tr>
<th>Glu1</th>
<th>Glu2</th>
<th>Glu3 (min)</th>
<th>Glu4</th>
<th>Glu5</th>
</tr>
</thead>
<tbody>
<tr>
<td>pABAGlu</td>
<td>7.3</td>
<td>13</td>
<td>22</td>
<td>26.7</td>
</tr>
<tr>
<td>PteGlu</td>
<td>7.7</td>
<td>14.7</td>
<td>21.3</td>
<td>26</td>
</tr>
<tr>
<td>H4PteGlu</td>
<td>7.0</td>
<td>13.7</td>
<td>20.7</td>
<td>25.7</td>
</tr>
<tr>
<td>H6PteGlu</td>
<td>7.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5-formyl-H4PteGlu</td>
<td>7.3</td>
<td>14.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5,10-methylene-H4PteGlu</td>
<td>7.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10-formyl-H4PteGlu</td>
<td>6.7</td>
<td>13.7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5-methyl-H4PteGlu</td>
<td>7.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Methotrexate Glu</td>
<td>10.7</td>
<td>16.6</td>
<td>22.3</td>
<td>26</td>
</tr>
</tbody>
</table>

—, compound unavailable for analysis.
due to equivalent changes in the 10-formyl-\(H_4\)PteGlu and \(H_4\)PteGlu pools, with little change in the detectable amount of the 5-methyl-\(H_4\)PteGlu pool. 5,10-Methylene \(H_4\)PteGlu was detectable only in the breast cells and remained below the limits of detection in the colon cells at all concentrations of 5-formyl-\(H_4\)PteGlu. In an effort to more accurately quantify this critical folate pool, we used two separate assay systems, including the HPLC and enzyme binding assays. Both assay systems provided similar results in that neither was able to detect 5,10-methylene \(H_4\)PteGlu in the colon cell line (detection limit, \(\sim 0.5 \) pmol/mg for both assays; Ref. 15), but both detected similar quantities in the breast cell line. Baseline levels of 5,10-methylene \(H_4\)PteGlu in the breast cell line were 1.8 (5.0 pmol/mg corrected for recovery) and 3.4 pmol/mg cytosolic protein (4.4 pmol/mg corrected for recovery) for the HPLC and binding assays, respectively. The 5,10-methylene \(H_4\)PteGlu pool expanded by a factor of 9.9 from 1.8 to 17.9 pmol/mg cytosolic protein in the breast cell line and to 10 \(\mu\)M 5-formyl-\(H_4\)PteGlu compared to these cells grown in 100 \(\mu\)M 5-formyl-\(H_4\)PteGlu as illustrated by the inset in Fig. 2. Intermediate exposure to 1, 5, and 10 \(\mu\)M 5-formyl-\(H_4\)PteGlu resulted in increases of 1.5-, 2.7-, and 3.4-fold compared to cells grown at the lowest concentration.

As illustrated in Fig. 3 (A and B), we investigated the metabolism of 5-formyl-\(H_4\)PteGlu in the two cell lines over a 24-h period and during a subsequent 24-h drug-free phase following the removal of labeled 5-formyl-\(H_4\)PteGlu from the media. In these experiments, we chose a constant 5-formyl-\(H_4\)PteGlu exposure concentration of 10 \(\mu\)M. We found that metabolism to the various folate forms was dependent on the exposure time up to 24 h. Following removal of the 5-formyl-\(H_4\)PteGlu, there was a rapid efflux of a large portion of the labeled intracellular folates over the first 2 h followed by a slower efflux phase. Since the metabolism of 5-formyl-\(H_4\)PteGlu appeared to be time and dose dependent in both the MCF-7 breast and HCT 116 colon cells, we compared the quantities of the various intracellular folates after a high-dose (10 \(\mu\)M) but brief exposure (2 h) to a low-dose (1 \(\mu\)M) but longer (24 h) exposure (Fig. 4). We found that the total intracellular labeled folate and the relative distribution of the various forms were similar for the two conditions in both cell lines, suggesting that prolonged exposure to low 5-formyl-\(H_4\)PteGlu concentration results in a similar distribution of folate forms compared to a short, high-dose exposure.

Polyglutamation is an important determinant of folate affinity for thymidylate synthase and intracellular retention. Therefore, we investigated the rate and extent of polyglutamation during exposure to both 1 and 10 \(\mu\)M 5-formyl-\(H_4\)PteGlu in the colon cell line and to 10 \(\mu\)M 5-formyl-\(H_4\)PteGlu in the breast line. The cells were exposed for various time points up to 24 h and for 2, 6, and 24 h following the removal of the labeled 5-formyl-\(H_4\)PteGlu from the media. We found that polyglutamation was time dependent in both cell lines with continuous increases, particularly for the higher chain lengths, for up to 24 h as shown in Table 2. In the HCT 116 colon cell line, exposure to the higher folate concentration resulted in similar or greater amounts of the polyglutamate forms, with the exception of the pentaglutamate after 6 and perhaps 2 h of exposure. These brief, higher-dose exposures resulted in a trend toward decreases in the absolute amounts of the higher polyglutamate forms compared to the lower-dose exposures. The higher polyglutamates (Glu3–Glu5) became prevalent only after the longer exposures and represented a substantial fraction of the total labeled intracellular folate pool in the 24-h exposures to either 1 or 10 \(\mu\)M 5-formyl-\(H_4\)PteGlu in both cell lines. A 24-h exposure to 1 \(\mu\)M 5-formyl-\(H_4\)PteGlu in the colon line resulted in the higher polyglutamate forms accounting for almost 90% of the total labeled intracellular folates. For the 24-h labeling period we found the pentaglutamate form to be the highest polyglutamate state detected in both cell lines. During the drug-free period, we found that the intracellular half-life increased exponentially with increasing polyglutamate tail length as illustrated in Fig. 5. These half-lives for each of the polyglutamate states represent the net effect of synthesis, degradation, and cellular efflux. The intracellular half-lives were similar in the two cell lines and measured 131, 143, 441, and 1167 min for the di-, tri-, tetra-, and pentaglutamates, respectively, in the HCT 116 colon cell line. The intracellular half-life for the total folate pool was similar for the two lines as well and measured 550 min. As noted in Fig. 4, prolonged exposures to low concentrations of 5-formyl-\(H_4\)PteGlu result in a similar one-carbon substituted folate distribution when compared to brief, high-dose exposures. Polyglutamation to the higher
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Polylglutamates states appears to be strongly favored by the former conditions. Fig. 6 illustrates a greater than 5-fold increase in the absolute quantity of intracellular Glu3–Glu5 with prolonged exposures compared to the 2-h exposures suggesting a benefit for the prolonged schedule even with a low concentration of 5-formyl-H4PteGlu.

Since the biologically inactive (6R) isomer of 5-formyl-H4PteGlu has a relatively long serum half-life and represents the predominant folate in the serum shortly after i.v. drug administration in patients, we investigated the effect of (6R)-5-formyl-H4PteGlu on the metabolism of (6S)-5-formyl-H4PteGlu to the various one-carbon substituted folate and polyglutamate states. We compared the formation of the intracellular folate forms after a fixed exposure of pure (6S)-5-formyl-H4PteGlu to metabolism following exposures to increasing ratios (up to 20:1) of (6R)- to (6S)-5-formyl-H4PteGlu in the HCT 116 colon cells. As illustrated in Fig. 7, the relative distribution and amounts of each of the one-carbon substituted folate forms were similar despite the addition of (6R)-5-formyl-H4PteGlu; however, the polyglutamates appeared to be minimally decreased in the HCT 116 cells exposed to increasing ratios (up to 20:1) of (6R)- to (6S)-5-formyl-H4PteGlu for a 24-h period. The decreases noted were principally apparent for the lower polyglutamate forms.

DISCUSSION

This report illustrates that the intracellular metabolism of 5-formyl-H4PteGlu to the various one-carbon substituted compounds, and their polyglutamate forms in human breast and colon cancer cell lines is both a time- and dose-dependent process. The metabolism of 5-formyl-H4PteGlu into the various folate compartments in the dose range studied was not a saturable process as evidenced by the absence of detectable intracellular folates during and after exposure to 10 μM 5-formyl-H4PteGlu in human colon and breast cancer cells. The cells were grown in the same manner as described in Fig. 2. The cells were exposed to 10 μM (6R,S)-5-formyl-H4PteGlu for 2, 6, and 24 h, after which they were washed two times in ice-cold phosphate-buffered saline and harvested. Cells exposed for 24 h were also washed with phosphate-buffered saline two times, new drug-free medium was added, and the cells were allowed to incubate for an additional 2, 6, or 24 h and then were harvested as described above. After harvesting, the labeled folates were separated and quantitated by HPLC. A, folate profiles found in the breast cells; B, folate profiles found in the colon cells. Points, mean of 3–5 independent experiments. SE for each point ranged from 15 to 30%.

Fig. 3. Intracellular folates during and after exposure to 10 μM 5-formyl-H4PteGlu in human colon and breast cancer cells. The cells were grown in the same manner as described in Fig. 2. The cells were exposed to 10 μM (6R,S)-5-formyl-H4PteGlu for 2, 6, and 24 h, after which they were washed two times in ice-cold phosphate-buffered saline and harvested. Cells exposed for 24 h were also washed with phosphate-buffered saline two times, new drug-free medium was added, and the cells were allowed to incubate for an additional 2, 6, or 24 h and then were harvested as described above. After harvesting, the labeled folates were separated and quantitated by HPLC. A, folate profiles found in the breast cells; B, folate profiles found in the colon cells. Points, mean of 3–5 independent experiments. SE for each point ranged from 15 to 30%.

Fig. 4. Effect of 5-formyl-H4PteGlu exposure time and concentration on intracellular folate pools. The HCT 116 colon cells and MCF-7 breast cells were grown according to the methods described for Fig. 2. The cells were exposed to 10 μM 5-formyl-H4PteGlu for 2 h and 1 μM 5-formyl-H4PteGlu for 24 h. The cells were washed two times with ice-cold phosphate-buffered and harvested. The folate pools were then separated and quantitated by HPLC as described above. Top, effect of the various doses and schedules on the breast cell folate metabolism; bottom, effect on the colon cell. Columns, mean of 3–5 independent experiments. The SE was <15% and <40% for each 2- and 24-h point, respectively.
cellular labeled 5-formyl-H₄PteGlu. This finding is in contrast to a report illustrating saturable intracellular metabolism of 5-formyl-H₄PteGlu in a human leukemia (CCRF-CEM) cell line (growing in 2.3 μM folate) exposed for 4 h to 10 and 100 nM 5-formyl-H₄PteGlu, while in the colon cancer cells, 5-methyl-H₄PteGlu was the predominant metabolite. 5,10-Methylene-H₄PteGlu was detectable only in the breast cancer cells, where it represented 5% of the total intracellular folates at the end of a 24-h exposure. In our studies, metabolism to the higher polyglutamate forms by folylpolyglutamate synthetase appeared to be a saturable process. The total amount of the higher polyglutamates (pentaglutamate) formed by up to 4-fold in a human colon xenograph model exposed to a 50-fold dose range (20–1000 mg/m²) of 5-formyl-H₄PteGlu (19). Romanini et al. (21) reported 5- and 11-fold increases in 5,10-methylene-H₄PteGlu in a human leukemia (CCRF-CEM) cell line (growing in 2.3 μM folate) exposed for 4 h to 10 and 100 μM 5-formyl-H₄PteGlu, respectively.

The washout phase following a 24-h 5-formyl-H₄PteGlu exposure was characterized by the loss of a substantial fraction of the intracellular folates over the first 2 h followed by a slower efflux phase. The slow terminal phase appeared to be due to the generation of folate polyglutamate forms, which have a prolonged intracellular half-life (vide infra). While other investigators have noted the rapid conversion of 5-formyl-H₄PteGlu to the polyglutamated state, our finding of a slow terminal half-life in these experiments is in contrast with reported studies using murine models showing a rapid return of intratumoral folates to baseline levels as the serum concentration of the folate falls (20). Polyglutamate formation in both the breast and colon cancer cell lines occurred continuously over time with the higher polyglutamate forms, particularly the pentaglutamate, accounting for the majority of the labeled intracellular folates at the end of a 24-h exposure. In our studies, metabolism to the higher polyglutamate forms by folylpolyglutamyl synthetase appeared to be a saturable process. The total amounts of the higher polyglutamates were equivalent after 24-h exposures to both 1 and 10 μM 5-formyl-H₄PteGlu, while the 10 μM exposures were associated with markedly higher levels of the monoglutamates (Fig. 6). As has been noted by others, the present studies suggest a modest decrease in the absolute amount of the higher polyglutamates (pentaglutamate) formed.

### Table 2. Intracellular polyglutamation of folates in the HCT 116 colon cells and the MCF-7 breast cells after exposure to labeled 5-formyl-H₄PteGlu

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Glu1</th>
<th>Glu2</th>
<th>Glu3</th>
<th>Glu4</th>
<th>Glu5</th>
<th>Total</th>
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</thead>
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<tr>
<td><strong>MCF-7 breast cells (10 μM 5-formyl-H₄PteGlu)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uptake</td>
<td>2</td>
<td>77.6 ± 20</td>
<td>4.7 ± 3.5</td>
<td>3.3 ± 2.1</td>
<td>3.6 ± 2.1</td>
<td>15.4 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>92 ± 18</td>
<td>10.0 ± 9.9</td>
<td>6.8 ± 2.7</td>
<td>9.6 ± 2.7</td>
<td>22.5 ± 9.2</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>104 ± 40</td>
<td>20.4 ± 3.4</td>
<td>18.5 ± 0.71</td>
<td>32.3 ± 9.3</td>
<td>121.5 ± 19</td>
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<tr>
<td>Washout</td>
<td>2</td>
<td>18 ± 9.4</td>
<td>6.1 ± 3.0</td>
<td>13.4 ± 2.7</td>
<td>15.9 ± 2.7</td>
<td>80.2 ± 29</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.8 ± 1.8</td>
<td>1.5 ± 2.2</td>
<td>21 ± 2.9</td>
<td>9.6 ± 0.92</td>
<td>67 ± 15</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>7 ± 5.7</td>
<td>3.3 ± 3.4</td>
<td>3.2 ± 4.4</td>
<td>6.7 ± 5.7</td>
<td>85.5 ± 13</td>
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<td><strong>116 colon cells (10 μM 5-formyl-H₄PteGlu)</strong></td>
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<tr>
<td>Uptake</td>
<td>2</td>
<td>60.5 ± 45</td>
<td>16.3 ± 2.1</td>
<td>8.8 ± 1.1</td>
<td>4.9 ± 1.4</td>
<td>3.8 ± 4.0</td>
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<td></td>
<td>6</td>
<td>44 ± 20</td>
<td>13.6 ± 11</td>
<td>5.8 ± 3.9</td>
<td>4.2 ± 1.9</td>
<td>4.9 ± 5.7</td>
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<tr>
<td></td>
<td>24</td>
<td>84.5 ± 39</td>
<td>25.1 ± 13</td>
<td>27.5 ± 14</td>
<td>34.5 ± 14.2</td>
<td>84.3 ± 62</td>
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<tr>
<td>Washout</td>
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<td>24.4 ± 3.2</td>
<td>10 ± 9.5</td>
<td>19 ± 9.6</td>
<td>18 ± 4.9</td>
<td>73.8 ± 27</td>
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<td></td>
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<td>9 ± 4.6</td>
<td>4.0 ± 2.2</td>
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<td></td>
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<td>11.0 ± 7.1</td>
<td>3.5 ± 3.5</td>
<td>7.2 ± 0.42</td>
<td>20 ± 9.7</td>
<td>20 ± 22</td>
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<td><strong>116 colon cells (1 μM 5-formyl-H₄PteGlu)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Uptake</td>
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<td>15.3 ± 1.8</td>
<td>5.9 ± 0.43</td>
<td>6.4 ± 1.5</td>
<td>4.6 ± 1.5</td>
<td>11 ± 7.0</td>
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<td></td>
<td>6</td>
<td>13.5 ± 9.2</td>
<td>4.3 ± 2.4</td>
<td>6.6 ± 1.9</td>
<td>8.5 ± 1.9</td>
<td>45.1 ± 15.1</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>10.6 ± 3.6</td>
<td>4.3 ± 1.4</td>
<td>6.1 ± 1.6</td>
<td>8.9 ± 2.6</td>
<td>93.6 ± 28</td>
</tr>
<tr>
<td>Washout</td>
<td>2</td>
<td>3.8 ± 2.4</td>
<td>4.1 ± 2</td>
<td>4.8 ± 3.4</td>
<td>5.0 ± 1.6</td>
<td>88.5 ± 43</td>
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<tr>
<td></td>
<td>6</td>
<td>1.5 ± 0.9</td>
<td>0.5 ± 0.27</td>
<td>0.62 ± 0.7</td>
<td>1.1 ± 1.8</td>
<td>66.7 ± 17</td>
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<tr>
<td></td>
<td>24</td>
<td>0.45 ± 0.83</td>
<td>0.36 ± 0.26</td>
<td>0.38 ± 0.29</td>
<td>0.24 ± 0.27</td>
<td>35.6 ± 16</td>
</tr>
</tbody>
</table>
METABOLISM OF 5-FORMYL TETRAHYDROFOLATE

exposure time rather than to exposure dose and may be optimized by prolonged infusion schedules, even to relatively low 5-formyl-H₄PteGlu concentrations. Prolonged exposure to 5-formyl-H₄PteGlu resulted in a greater degree of polyglutamation (Fig. 6) without compromising metabolism into the various one-carbon substituted pools even at low doses (Fig. 4). Previous studies have shown that the higher polyglutamate forms display an increased affinity to thymidylate synthase (16, 17). Moreover, polyglutamation markedly prolongs the intracellular half-life of folates. In the present study, we noted an exponential increase in intracellular half-life of the folates as a function of their polyglutamate chain length. The half-life measured in these studies represents the net effect of anabolism by poly(polyglutamyl) synthetase, catabolism by hydrolase, and transmembrane transport of the various polyglutamate forms. The half-life measured in the present study for mono- (71 min) and pentaglutamated (1167 min) forms compares favorably with the half-life of methotrexate mono- (65 min) and pentaglutamate (1599 min) reported by Kennedy et al. (32) using a human breast cancer cell line (MDA MB.436). The intermediate folate polyglutamates had intermediate half-lives, as did the methotrexate polyglutamates. However, the reported half-life for the intermediate methotrexate polyglutamates appeared to be slightly longer (2-3-fold) compared to the intermediate folate polyglutamates reported in the present study. This difference may reflect a difference in the kinetic handling of folate versus methotrexate polyglutamates by both the anabolic and catabolic enzymes. Cell line differences probably do not account for the differences, since methotrexate polyglutamate half-life values calculated from MCF-7 cells are similar to those determined in the MDA MB.436 cells (33). Furthermore, our studies in the

in the colon cells after brief exposures to the higher 5-formyl-H₄PteGlu concentration (10 μM) compared to the amount formed after exposure to a lower concentration (1 μM) (19). This phenomenon may be the result of competition for folypolyglutamyl synthetase by the higher levels of mono- and diglutamates in cells exposed to the higher 5-formyl-H₄PteGlu concentrations. Cichowicz and Shane (31) have demonstrated that for folate substrates the mono- and diglutamated forms are generally the preferred substrates for mammalian folylpolyglutamyl synthetase, with the higher polyglutamates being less effective substrates (31). The relevance of this modest change in polyglutamate profile with increasing 5-formyl-H₄PteGlu doses is unclear, given the negligible differences in affinity to thymidylate synthase for polyglutamates greater than the diglutamate level (17). However, prolonged exposure to even low 5-formyl-H₄PteGlu concentrations strongly favors metabolism to the higher polyglutamates forms as illustrated in Fig. 6. It is of interest that in colon cancer cells given a similar area under the curve exposure (concentration x time), the absolute amount of the higher polyglutamates is 5-fold greater in cells exposed for 24 h to 1 μM versus cells treated for 2 h with 10 μM 5-formyl-H₄PteGlu. This observation suggests that the efficacy of 5-formyl-H₄PteGlu treatment may be more sensitive to total exposure time rather than to exposure dose and may be optimized by prolonged infusion schedules, even to relatively low 5-formyl-H₄PteGlu concentrations. Prolonged exposure to 5-formyl-H₄PteGlu resulted in a greater degree of polyglutamation (Fig. 6) without compromising metabolism into the various one-carbon substituted pools even at low doses (Fig. 4). Previous studies have shown that the higher polyglutamate forms display an increased affinity to thymidylate synthase (16, 17). Moreover, polyglutamation markedly prolongs the intracellular half-life of folates. In the present study, we noted an exponential increase in intracellular half-life of the folates as a function of their polyglutamate chain length. The half-life measured in these studies represents the net effect of anabolism by poly(polyglutamyl) synthetase, catabolism by hydrolase, and transmembrane transport of the various polyglutamate forms. The half-life measured in the present study for mono- (71 min) and pentaglutamated (1167 min) forms compares favorably with the half-life of methotrexate mono- (65 min) and pentaglutamate (1599 min) reported by Kennedy et al. (32) using a human breast cancer cell line (MDA MB.436). The intermediate folate polyglutamates had intermediate half-lives, as did the methotrexate polyglutamates. However, the reported half-life for the intermediate methotrexate polyglutamates appeared to be slightly longer (2-3-fold) compared to the intermediate folate polyglutamates reported in the present study. This difference may reflect a difference in the kinetic handling of folate versus methotrexate polyglutamates by both the anabolic and catabolic enzymes. Cell line differences probably do not account for the differences, since methotrexate polyglutamate half-life values calculated from MCF-7 cells are similar to those determined in the MDA MB.436 cells (33). Furthermore, our studies in the

![Fig. 5. Intracellular half-life of the folate polyglutamates in the human colon cells. The intracellular half-life for each of the polyglutamate forms was calculated by fitting the data shown in Table 2 to nonlinear equations using Cricket Graph software package (Cricket Software, Malvern, PA). The data used for each fit represented the baseline value and 3 time points (2, 6, and 24 h) following removal of 5-formyl-H₄PteGlu from the media. Each of the 4 time points used to calculate the half-lives represented 3-6 independent experiments. Points, mean values from all the experiments; bars, SE. Curve, best exponential fit (r² = 0.93).](image1)

![Fig. 6. Effect of 5-formyl-H₄PteGlu exposure time and concentration on the polyglutamation of folates in human colon cells. The HCT 116 colon cells were grown according to methods described in Fig. 2. The cells were exposed to 1 and 10 μM 5-formyl-H₄PteGlu for 2 and 24 h. After the appropriate exposure, the cells were harvested and the polyglutamated folates were separated and quantitated by HPLC as previously described. Columns, effect of the different 5-formyl-H₄PteGlu exposure concentrations and durations on polyglutamation in the colon cells, mean of 3 independent experiments.](image2)

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The final issue addressed by these studies was the influence of the biologically inactive isomer of 5-formyl-H$_4$PteGlu on the uptake and intracellular metabolism of the biologically active isomer. Studies by Bertrand and Jolivet (35) suggest that the inactive isomer may interfere with membrane transport of the active isomer. However, their studies demonstrated no effect of the inactive isomer on the ability of the active folate to augment the cytotoxicity of 5-fluorouracil in the leukemic CCRF-CEM cell line. Studies performed by Schilsky et al. (36) on the uptake and activity of the two stereoisomers of 5-formyl-H$_4$PteGlu have shown that both the (6S) and (6R) isomers are taken up by HT-29 colon cells in a dose-dependent fashion up to 5 µM. However, they reported an approximately 30-fold decreased accumulation of the (6R) isomer compared with the (6S) isomer at equivalent extracellular concentrations. They also observed no inhibition of (6S) uptake with ratios of 1000:1 of (6R) to (6S) isomer (36). The present studies identified little effect of the inactive (6R) isomer on the metabolism of the active form to the various one-carbon substituted compounds or their polyglutamated forms at ratios of up to 20:1 inactive:active isomer. However, with increasing concentrations of the inactive isomer, we observed a modest decrease in the mono-, di-, and triglutamate forms. The apparent lack of effect of the inactive isomer on transport and/or metabolism of the active isomer may be due to lack of competition at the level of membrane transport and either poor affinity for the folate-dependent metabolic enzymes or insufficient intracellular concentrations of the inactive isomer to effectively compete for enzyme binding.

The present studies investigating the metabolism of 5-formyl-H$_4$PteGlu in human colon and breast cancer cell lines suggest that prolonged exposure may be the optimal delivery schedule in that it strongly favors polyglutamation of the folates to the higher levels. Clinical studies in patients with advanced colorectal carcinoma utilizing the daily schedule for 5 days have been the only reports associated with a significant prolongation of patient survival. A recent study utilizing this schedule demonstrated equal efficacy in patients treated with either 20 or 200 mg/m$^2$ day 5-formyl-H$_4$PteGlu for 5 days (7). A separate study using a weekly schedule comparing high- versus low-dose 5-formyl-H$_4$PteGlu demonstrated superior results with the high-dose schedule (8). These studies suggest that prolonged or repetitive exposure to even low doses of 5-formyl-H$_4$PteGlu rather than a single high-dose exposure may be a critical factor in clinical efficacy. Finally, these studies suggest that the inactive (6R) isomer of 5-formyl-H$_4$PteGlu may only minimally impact the metabolism of the active (6S) isomer in human tumor cells.

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Intracellular Metabolism of 5-Formyl Tetrahydrofolate in Human Breast and Colon Cell Lines

Donna M. Boarman and Carmen J. Allegra

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