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# DEOXYURIDYLATE EFFECTS ON THYMIDYLATE SYNTHASE–5-FLUORODEOXYURIDYLATE–FOLATE TERNARY COMPLEX FORMATION*

COLIN PAUL SPEARS,† ANDREA A. HAYES, ANTRANIK H. SHAHINIAN and PETER V. DANENBERG

Department of Medicine, University of Southern California Comprehensive Cancer Center, Los Angeles, CA 90033, U.S.A.

and

ROLAND FRÖSING and BENGT G. GUSTAVSSON

Department of Surgery, Östra Hospital, University of Göteborg, S41685 Göteborg, Sweden

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**Abstract**—The competitive basis and specificity of deoxyuridylate (dUMP)-mediated decreases in thymidylate synthase–5′-fluorodeoxyuridylate–folate (TS–FdUMP–folate) ternary complex formation at low concentrations of folates were investigated using charcoal isolation of protein-bound [$^3$H]FuUMP ligand. Reaction conditions used 0.02 μM TS (*Lactobacillus casei*) and 0.10 μM [$^3$H]FdUMP incubated for 10 min at 37° and pH 7.4. Decreases in counts below control (C) values in dUMP-added samples (S) were expressed as C/S ratios. At $CH_2$—$H_4PteGlu_1$ or $H_4PteGlu_1$ concentrations below 10 μM, highly linear relationships were found to exist between C/S value and dUMP concentrations, expressed as dUMP/FdUMP ratios. For $H_4PteGlu_1$, maximal C/S values for dUMP interference occurred at the lowest $H_4PteGlu_1$ concentrations, approaching the value of the TS–FdUMP binary complex. The efficiency of ternary complex formation by $H_4PteGlu_1$ was 28 ± 5% of $CH_2$—$H_4PteGlu_1$ values at concentrations below 1.0 μM. The protective effect of increasing $H_4PteGlu_1$ against dUMP interference resulted in a linear relationship between the logarithm of $H_4PteGlu_1$ concentration and the slope of dUMP interference (C/S vs dUMP/FdUMP). In contrast, the results with $CH_2$—$H_4PteGlu_1$ were biphasic. At concentrations of $CH_2$—$H_4PteGlu_1$ lower than 0.5 μM, C/S values were greater than those for binary complex alone, a result related to $CH_2$—$H_4PteGlu_1$ consumption based on [5-$^3$H]dUMP tritium-release studies. At concentrations of $CH_2$—$H_4PteGlu_1$ above 1.0 μM, however, dUMP interference was nearly abolished. Kinetic analysis of the data suggests that this effect of the 5,10-methylene moiety may result in part from positive allosteric effects of first site TS–FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex binding on acceleration of second site binding, in addition to slowed rates of dissociation. Other folylmonoglutamates showed relatively poor TS–[$^3$H]FdUMP–folate complex formation: at 500 μM folate, as a percentage of $CH_2$—$H_4PteGlu_1$ values, these were 29.6% for dihydrofolate, 7.5% for 5-$CH_3$—$H_4PteGlu_1$, 3.0% for CH=$H_4PteGlu_1$, 1.6% for folic acid, 1.1% for 5-CHO—$H_4PteGlu_1$ (leucovorin) and 0.9% for 10-CHO—$H_4PteGlu_1$. Inhibitory effects by dUMP were consistent with binary complex effects alone for these folates. Study of methotrexate, as the monoglutamate and the hexaglutamate, suggested that ternary complexes with dUMP are favored over those with FdUMP at high concentrations of the antifolate. Our results indicate that activation of leucovorin to over 0.5 μM in intracellular $CH_2$—$H_4PteGlu_1$ equivalents may be a requirement for achieving complete TS inhibition by FdUMP in the presence of excess concentrations of dUMP. Use of TS-[$^3$H]FdUMP ligation to measure intracellular $CH_2$—$H_4PteGlu_n$ concentrations may result in significant overestimates in the presence of excess $H_4PteGlu_n$.

The thymidylate synthase (TS‡) mechanism of cytotoxicity of 5-fluorouracil (FUra) and 5-fluorodeoxyuridine has attracted increased interest, because expansion of innately low intracellular concentrations of 5,10-methylenetetrahydropteroylglutamates ($CH_2$—$H_4PteGlu_n$) and other reduced folates by folate supplementation increases the cytotoxicities of these fluoropyrimidines by 2- to 50-fold [1–4]. This presumably is a result of increased stability of TS–5-fluorodeoxyuridylate (FdUMP)–$CH_2$—$H_4PteGlu_n$ ternary complexes. Addition of 5-CHO—$H_4PteGlu_1$ (folinic acid or leucovorin) to FUra treatment of human colorectal adenocarcinomas, based on this rationale, results in substantial increases in response rates [5]. Kinetic studies have shown that TS–FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex formation occurs by an ordered, sequential mechanism, with stoichiometric quantities of FdUMP binding first and $CH_2$—$H_4PteGlu_1$ second

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† Author to whom correspondence should be addressed: Cancer Research Laboratory, Room 206, University of Southern California, Comprehensive Cancer Center, 1303 North Mission Road, Los Angeles, CA 90033, U.S.A.

‡ Abbreviations: FUra, 5-fluorouracil; dUMP, 2′-deoxyuridylate; $H_4PteGlu_1$, tetrahydropteroylmonoglutamate; $CH_2$—$H_4PteGlu_1$, 5,10-methylenetetrahydropteroylmonoglutamate; 5-CHO-$H_4PteGlu_1$, 5-formyltetrahydropteroylmonoglutamate (leucovorin or folinic acid); CH=$H_4PteGlu_1$, 5,10-methenyltetrahydropteroylmonoglutamate; TS, thymidylate synthase, $CH_2$—$H_4PteGlu_n$, dUMP C-methyltransferase (EC 2.1.1.45); FdUMP, 5-fluorodeoxyuridylate; and MTX, methotrexate.

[6–9]. Dissociation of the complex is enzyme-catalyzed and kinetically first-order, with $CH_2$—$H_4PteGlu_1$ departing first in a reverse sequence. FdUMP dissociation from enzyme is slowed markedly by excess $CH_2$—$H_4PteGlu_1$ as a result of the obligatory binding sequence [8–10], perhaps more so with mammalian than bacterial TS [9], with an upper limit for this effect in the low millimolar range. TS–FdUMP binary complex formation, albeit covalent, has a dissociation constant approximately a million-fold weaker than the ternary complex [8, 10–13].

An important determinant of TS–FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex formation that has received only limited kinetic evaluation [7–9, 14, 15] is 2′-deoxyuridylate (dUMP), the natural substrate for TS for conversion to TMP. Initial TS–FdUMP binary complex formation is slowed markedly by dUMP in concentrations in excess of its $K_s$, which is about 0.4 μM [8]. The consequent effects on ternary complex formation were used by Myers *et al.* [16] as the basis of a spectrophotometric assay of intracellular dUMP levels. Possibly because of fairly long incubation times, during which $CH_2$—$H_4PteGlu_n$ may be expected to oxidize spontaneously, dUMP levels were likely to have been overestimated, on comparison with more specific assays [17]. Nonetheless, we have found that dUMP levels are high enough in tumors [18–20] to cause effects that have been described as multiplicative [9] with low $CH_2$—$H_4PteGlu_n$ concentrations on limiting inhibition of TS by FdUMP based on decreases in rates of ternary complex formation.

Estimates of the optimal intracellular $CH_2$—$H_4PteGlu_1$ level for stabilization of TS–FdUMP–folate ternary complexes can vary somewhat [1–3, 14], possibly related to variations in dUMP concentrations. Measurement of tissue $CH_2$—$H_4PteGlu_n$ levels, by trapping this folate on addition of excess bacterial TS and [$^3$H]FdUMP, offers a method [21–23] for documenting increases in $CH_2$—$H_4PteGlu_n$ after leucovorin supplementation. However, elevated dUMP concentrations could decrease TS–[$^3$H]FdUMP–$CH_2$—$H_4PteGlu_n$ ternary complex formation by increased conversion of $CH_2$—$H_4PteGlu_n$ to dihydrofolate as well as by competition with [$^3$H]FdUMP for initial TS–nucleotide binary complex formation. These effects are quantitated in the present analysis, which shows that, at concentrations of $CH_2$—$H_4PteGlu_1$ below 0.5 μM, nearly any dUMP in excess of FdUMP caused severe interference with ternary complex formation, yet above 1.0 μM the effects of even large concentrations of dUMP on TS inhibition by FdUMP were negligible.

## MATERIALS AND METHODS

*Materials.* TS was the enzyme from methotrexate-resistant *Lactobacillus casei*, isolated and purified to a specific activity of 3.5 units/mg protein, based on spectrophotometric assay [17]. Stock TS was stored at 4° in 20 mM 2-mercaptoethanol and 5 mM potassium phosphate buffer, pH 6.8, at a concentration of 5.0 units/ml (or 21 pmol TS/μl), and dialyzed against fresh buffer ever 3–4 weeks to remove disulfides. (6*S*)-Tetrahydropteroylmonoglutamate ($H_4PteGlu_1$) was prepared as described [17] and stored under nitrogen in sealed glass ampoules at −20°. (6*R*)-5,10-Methylenetetrahydropteroylmonoglutamate ($CH_2$—$H_4PteGlu_1$) was prepared on the day of use by addition of 50 μl of 1 M sodium ascorbate, pH 6.5, 1.5 μl of 37% $CH_2O$, 14 μl of 2-mercaptoethanol, and 0.935 ml of 0.2 M Tris–HCl, pH 7.0, to 4 μmol of $H_4PteGlu_1$, followed by a 10-min incubation at 23° and storage at 4° in the dark. Dilutions of $CH_2$—$H_4PteGlu_1$ were made at 4° in Buffer A (0.2 M Tris–HCl buffer, pH 7.4, containing 20 mM 2-mercaptoethanol, 20 mM $CH_2O$, and 100 mM sodium ascorbate). Nucleotides (dUMP, FdUMP, TMP, CMP, UMP, dCMP, dAMP, dGMP), folic acid, dihydrofolate, 5-methyl-tetrahydropteroylmonoglutamate, 10-formyl-tetrahydropteroylmonoglutamate, and FdUrd were from the Sigma Chemical Co. (St Louis, MO) and used as received. (6*R*,*S*)-5-Formyl-tetrahydropteroylmonoglutamate (folinic acid, 5-CHO—$H_4PteGlu_1$, leucovorin as the calcium salt) and methotrexate were Lederle products. Methotrexate (MTX) hexaglutamate was purchased from American Radiolabeled Chemicals (St Louis, MO). Synthesis of 5,10-methenyl-tetrahydropteroylmono-glutamate (CH=$H_4PteGlu_1$) from 5-CHO—$H_4PteGlu_1$, was done by the method of Rabinowitz [24]. Buffer A without formaldehyde was used for dilution of all other folates. [6-$^3$H]FdUMP (18–20 Ci/mmol) and [5-$^3$H]dUMP (18–20 Ci/mmol) were from Moravek Biochemicals (Brea, CA). Charcoal, 3% suspension, was prepared by dilution of 10% (w/v) neutral $H_2O$-washed activated charcoal containing 2.5% albumin and 0.25% dextran in HCl (final concentration 0.2 N). Stock solutions of [$^3$H]FdUMP were chromatographed on 3.0-cm $NH_4HCO_3$-form columns [17] every 4–6 weeks for removal of radiolysis products.

*Thymidylate synthase and $CH_2$—$H_4PteGlu_n$ assays.* To a series of conical-tipped plastic test tubes at 0° were added 50 μl of dUMP in water, 5–20 pmol of [$^3$H]FdUMP in 50 μl water, and 50 μl of $CH_2$—$H_4PteGlu_1$ in 0.2 M Tris–HCl buffer, pH 7.4, containing 20 mM 2-mercaptoethanol and 100 mM sodium ascorbate. The contents were mixed, and then 0–20 pmol of *L. casei* TS in 50 μl of Buffer B [0.2 M Tris–HCl buffer, pH 7.4, containing 20 mM 2-mercaptoethanol and 0.2% (w/v) bovine serum albumin] was added to a total volume of 200 μl. Incubations were carried out at 37° for 10 min, protected from light, and then protein-bound radioactivity was isolated by addition of 1.0 ml of ice-cold 3% (w/v) albumin- and dextran-coated charcoal in 0.2 N HCl, followed by mixing, then centrifugation at 3000 *g* for 20 min [17]. To 0.8-ml aliquots of the supernatant fractions was added 20 ml of RIA-SolveII (Research Products International, Mount Prospect, IL). Radioactivity was determined using a Beckman LS-9000 scintillation counter, using 10 min counts. Controls routinely included the conditions of no enzyme, no dUMP, or no folate for determination of radioactivity due to TS–[$^3$H]FdUMP binary complex formation. Binary complex (no folate) backgrounds for given concentrations of

dUMP were subtracted from all ternary complex results.

The rate of release of $^3H_2O$ from [5-$^3$H]dUMP was used to study TS activity [25] under incubation conditions identical to those for TS–[$^3$H]FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex formation. In sequence at 4° were added 0.4 μCi of [$^3$H]dUMP, either 20 pmol (sp. act. 20 Ci/mmol) or 800 pmol (sp. act. 0.5 Ci/mmol); non-radiolabeled FdUMP, 20 pmol, or $H_2O$ blank; $CH_2$—$H_4PteGlu_1$, or $H_4PteGlu_1$, 5–625 pmol or buffer alone; then TS, 4 pmol, or buffer B alone, was added, in a total reaction volume of 200 μl. Following a 10-min incubation at 37°, 1 ml of ice-cold 3% (w/v) albumin- and dextran-coated charcoal (0.2 N in HCl) was added for determination of radioactivity present in 3000 *g* supernatant fractions.

## RESULTS

*Effects of dUMP on TS–FdUMP binary complex formation.* Acidified charcoal was useful for separation of free from protein-bound [$^3$H]FdUMP, enabling study of the effects of dUMP on TS–[$^3$H]FdUMP binary complex formation, not observed by nitrocellulose filtration in an earlier study [10]. In agreement with others [12, 13], who did use nitrocellulose separation, somewhat greater yields of binary complexes were obtained with neutral conditions, which we found, however, also increased the experimental error with lowered yield of ternary complexes. No correction for filtration efficiency [15] of charcoal was made, based on the reasoning that differences between ionic and covalent complexes in this regard are difficult to quantitate [13]. Blanks in the absence of enzyme averaged 2986 ± 37 dpm (N = 7), less than 0.4% of radioactivity initially present.

The relationship between yield of binary complex and concentration of TS between 0.007 and 0.213 μM, at a fixed concentration of [$^3$H]FdUMP (0.167 μM), was determined with and without dUMP (4.0 μM). At the highest concentration of enzyme, in the absence of dUMP, the maximum yield of acid charcoal-isolable [$^3$H]FdUMP was 1.48% of ternary complex results; the increase in binary complex with higher TS appeared to show saturation kinetics, with the ratio of (TS-bound FdUMP)/(TS) decreasing nearly linearly from 15 to 7 fmol of [$^3$H]FdUMP bound per pmol of TS toward the higher concentrations of TS. Thus, there was decreasing affinity of TS for FdUMP with increasing concentrations of TS binding sites greater than FdUMP. Measured rates of TS–FdUMP binary complex formation using 0.02 μM TS and 0.10 μM FdUMP suggested two first-order processes, with an initial $k_{on}$ of 0.58/min (half-life, 1.2 min), with a second, slower phase of binding ($k_{on}$ = 0.094/min, half-life 7.4 min) apparent after 5 min. This probably reflected sequential binding and isomerization steps [26]. The simultaneous presence of dUMP decreased TS–[$^3$H]FdUMP binary complex formation, with a greater relative effect at lower concentrations of TS binding sites. For example, at 0.029 μM TS, the ratio of binary complex formation in the absence (C) or presence (S) of dUMP, or C/S value, was linearly related to the ratio of dUMP/FdUMP:

$$(C/S) - 1 = 0.13(dUMP/FdUMP). \quad (1)$$

The C/S intercept of this relationship, 1.00, was decreased slightly (0.96) when TS binding sites were in excess over FdUMP, suggestive of slight positive homotropic effects of dUMP toward paradoxically increasing FdUMP binding. This interpretation is based on observations of differing binding affinities of the two sites, with initial high-affinity binding promoting subsequent second-site binding [8, 27]. Consistent with this interpretation, TS–[$^3$H]FdUMP binary complex formation versus TS concentration, in the presence of a 40-fold excess of dUMP over FdUMP, was highly linear, showing no trend toward saturation with increasing concentrations of TS. In this case, where dUMP was greatly in excess over all other ligands, the specific activity of protein-bound radioactivity was the same (2.4 fmol FdUMP/pmol TS) at all concentrations of TS.

Expressed as the C/S ratio, the net effect of dUMP showed increasingly severe interference with TS–[$^3$H]FdUMP binary complex formation with decreasing TS concentrations, exactly the opposite of observations with ternary complexes. This observation served as a check for showing the lack of contaminating $CH_2$—$H_4PteGlu_1$ in the binary complex values, which were used as blanks in quantitation of ternary complexes.

*Effects of dUMP on TS–FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex formation.* The time of incubation, 10 min, was chosen based on the following considerations. In the absence of dUMP, the initial velocity of TS–[$^3$H]FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex formation, using 0.5 μM folate, 0.02 μM [$^3$H]FdUMP, and 0.02 μM TS, was rapid ($k_{on} = 1.6 \times 10^8$/M/min), with a half-life of reaction of 1.6 min. Maximum formation occurred by 6 min, with a slow (11.4% decrease per hr) first-order loss of ternary complex occurring over a subsequent 1-hr incubation. With excess dUMP present (0.1 μM), maximum ternary complex formation also occurred by 6 min, with only a slightly greater loss of ternary complex (18.3% per hr), on continued incubation at 37°. The initial on-rate slowed in approximate proportion to the C/S values, $k_{on} = 0.5 \times 10^8$/M/min. Thus, C/S values were unaffected by time of incubation after 10 min, averaging 3.3 ± 0.1 up to 60 min, for 0.5 μM $CH_2$—$H_4PteGlu_1$.

Figure 1 shows the profound effects caused by dUMP in limiting TS–[$^3$H]FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex formation at low concentrations of $CH_2$—$H_4PteGlu_1$. In the absence of dUMP, half-maximal ternary complex formation occurred with a 0.1 μM concentration of the folate. Maximal, saturating levels of $CH_2$—$H_4PteGlu_1$ were only 10 μM, resulting in occupancy of all available TS binding sites at a ratio of [$^3$H]FdUMP:TS of about 1.8:1. For $CH_2$—$H_4PteGlu_1$ concentrations below 0.5 μM, a series of parallel isobars resulted from different dUMP concentrations. The log–log relationships were used to derive Eqn 2, applicable to concentrations of $CH_2$—$H_4PteGlu_1$ below 0.5 μM:

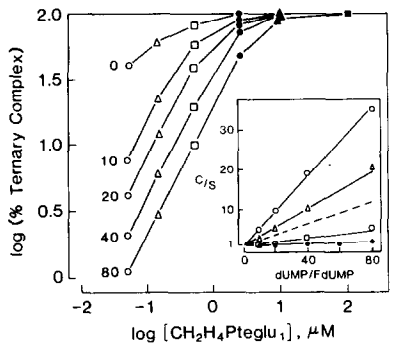


Fig. 1. *L. casei* TS-[$^3$H]FdUMP-$CH_2$—$H_4PteGlu_1$ ternary complex formation as a function of variable initial $CH_2$—$H_4PteGlu_1$ and dUMP concentrations. Reaction mixtures, incubated for 10 min at 37°, contained 0.10 mM [$^3$H]FdUMP and 0.023 μM TS in 0.2 M Tris–HCl buffer, pH 7.4. Prior to TS addition, $CH_2$—$H_4PteGlu_1$ in Buffer A was added to the final concentrations shown and dUMP was present at ratios of dUMP/FdUMP of 0, 10, 20, 40, and 80 (isobars). Results are given in terms of maximal yield of ternary complex (209,142 dpm) isolated by addition of acidified charcoal. The inset shows a replot of the data, based on ratios of control (C) divided by dUMP-added samples (S), or C/S values. The dashed line represents C/S values for the TS–FdUMP binary complex.

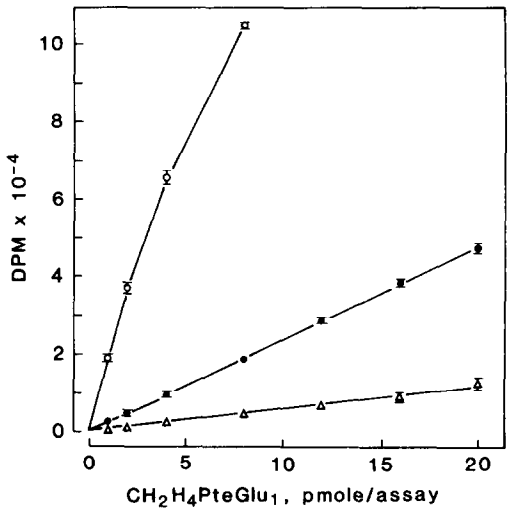


Fig. 2. Standard curves for $CH_2$—$H_4PteGlu_1$ assay, using 0.10 nM [$^3$H]FdUMP and 0.023 nM *L. casei* TS in 175 μl reaction volumes, and incubation at 37° for 10 min. Concentrations of dUMP were zero (○), 0.5 nM (●), and 2.0 nM (△). Average experimental standard deviations were smaller than the symbols shown, N = 3.

$$[CH_2\text{—}H_4PteGlu_1, \text{apparent}] = \frac{2.91[CH_2\text{—}H_4PteGlu_1]}{(dUMP/FdUMP) \times \log [CH_2\text{—}H_4PteGlu_1]} \quad (2)$$

where dUMP is expressed as the dUMP/FdUMP ratio. Essentially identical results were obtained repeating the experiment using a 4-fold lower concentration of FdUMP. The solution of Eqn 2 for $CH_2$—$H_4PteGlu_1$ is iterative. These relationships are more fully described in Fig. 4, discussed below.

Highly linear relationships emerged between dUMP/FdUMP ratios and C/S values, shown in the inset of Fig. 1. For a given dUMP/FdUMP ratio, standard curves for assay of $CH_2$—$H_4PteGlu_1$ at low concentrations resulted. The repeatability of such linearity at low $CH_1$—$H_4PteGlu_1$ concentrations also provided a basis for facile, sensitive assay of dUMP. The slope of this relationship was usefully steep at concentrations of $CH_2$—$H_4PteGlu_1$ below 0.5 μM. This assay of dUMP has several advantages over, and gives results similar to, the method using [$^{14}$C]formaldehyde [17], application of which will be reported elsewhere.

The slope of the dotted line, in the inset in Fig. 1, represents the results obtained for the TS–[$^3$H]FdUMP binary complex control. Thus, at $CH_2$—$H_4PteGlu_1$ concentrations below 0.5 μM the relative interference by dUMP in decreasing FdUMP-ternary complex formation was increasingly greater than can be explained on the basis of competition effects between the two nucleotides for initial binary complex formation.

Figure 2 shows representative examples of standard curves for $CH_2$—$H_4PteGlu_1$ assay. The slope of the standard curve without dUMP was 19 dpm/fmol of $CH_2$—$H_4PteGlu_1$, or 42% of stoichiometric binding of this folate (assuming 1.7 binding sites/TS molecule). Based on an FdUMP assay [17], the specific activity of our commercial [$^3$H]FdUMP may have been as low as 17 Ci/mmol, which would give a calculated binding efficiency of 49% for $CH_2$—$H_4PteGlu_1$. Linearity was observed below 23 nM (4 pmol/assay) $CH_2$—$PteGlu_1$ down to a detection limit of about 20 fmol (not shown) based on average standard deviations below 300 dpm. The presence of dUMP, at concentrations 5- and 20-fold in excess over FdUMP, did not increase scatter in the data, and resulted in fully linear standard curves over a wide range than $CH_2$—$H_4PteGlu_1$ concentrations alone, that began to show saturation effects above 20 nM.

*Effects of dUMP on TS-*[$^3$H]*FdUMP–$H_4PteGlu_1$ ternary complex formation.* At relatively high concentrations of $H_4PteGlu_1$, over 10 μM, charcoal-isolable ternary complex formation was at least 96.6% of values obtained with $CH_2$—$H_4PteGlu_1$ (Fig. 3). The isobars for yields of TS–FdUMP–$H_4PteGlu_1$ ternary complex at different ratios of dUMP/FdUMP were uniformly parallel. A replot of these data according to C/S values, shown in the inset for Fig. 3, shows that increasing $H_4PteGlu_1$ decreased the negative effects of dUMP on ternary complex formation at all concentrations of $H_4PteGlu_1$, over that of the TS-FdUMP binary complex control. A graph of log[$H_4PteGlu_1$] vs the slope of the C/S data (Fig. 4) showed near-linearity between these parameters.

In striking contrast, the result for $CH_2$—$H_4PteGlu_1$ (Fig. 4) showed an abrupt transition point below 0.5 μM for demonstrating marked increases in the antagonistic effects of dUMP. Above 0.5 μM, the curves for the two folates are parallel, with more powerful effects by covalently-bonding $CH_2$—$H_4PteGlu_1$ in abrogating dUMP antagonism. Below 0.5 μM $CH_2$—$H_4PteGlu_1$, the dUMP-me-

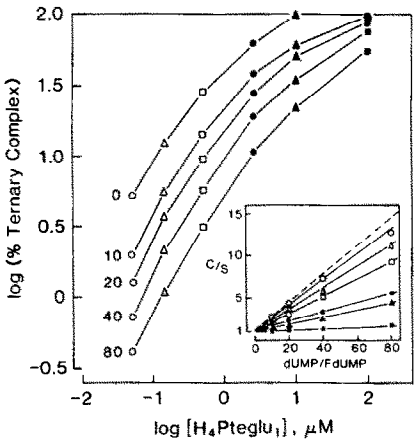


Fig. 3. TS–[$^3$H]FdUMP–$H_4PteGlu_1$ ternary complex formation as a function of variable initial $H_4PteGlu_1$ and dUMP concentrations. Isobars represent different dUMP/FdUMP ratios. Reaction conditions were exactly the same as for $CH_2$—$H_4PteGlu_1$ (Fig. 1) except that formaldehyde was omitted. Dimedone (5 mM) controls showed no effect. The inset shows replots of the data based on C/S values, with comparison made to the result for TS–FdUMP binary complex (dashed line). Relative to values for $CH_2$—$H_4PteGlu_1$, ternary complex formation at dUMP/FdUMP ratios of 0, 10, 20, 40, and 80, at the lowest $H_4PteGlu_1$ concentration (0.05 μM) was 14, 26, 31, 35, and 37%; at 0.5 μM, 28, 26, 26, 30 and 32%; and at 2.5 μM, 63, 44, 35, 27, and 22% respectively.

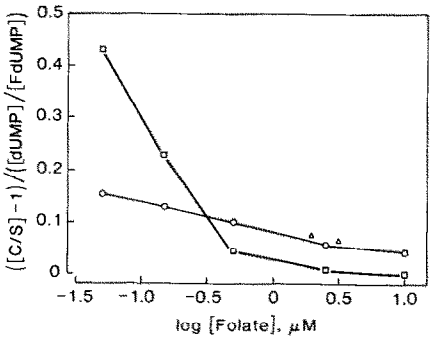


Fig. 4. Replots of the slopes from the inset of Figs 1 and 3 versus logarithm of micromolar folate concentration. The highly linear result for $H_4PteGlu_1$ (○) is asymptotic at the binary complex value. The relationship between dUMP/FdUMP concentration and decreases in yield of ternary complex for initial $CH_2$—$H_4PteGlu_1$ (□) concentration below 0.5 μM is given by Eqn 2 in the text. Data calculated from Table 4 of Ref. 10, using initial rate determinations by nitrocellulose isolation of TS–[$^3$H]FdUMP–$CH_2$—$H_4PteGlu_1$ are shown by (△).

diated decreases in TS–FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex formation may alternatively be represented by Eqn 2.

*Effects of dUMP on ternary complex formation by other pteroylmonoglutamates.* The relationship between folate concentration and charcoal-isolable TS–[$^3$H]FdUMP–folate ternary complex formation using incubations of 10 min and 30°, 0.03 μM [$^3$H]FdUMP and 0.1 μM enzyme are shown in Fig.

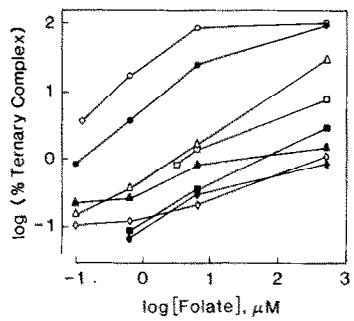


Fig. 5. TS–[$^3$H]FdUMP–folate ternary complex formation as a function of concentration of folate and maximum, 100% value for $CH_2$—$H_4PteGlu_1$, using 10-min incubations at 30° and charcoal isolation of protein-bound radioactivity. Comparative results are shown for $CH_2$—$PteGlu_1$ (○), $H_4PteGlu_1$ (●), dihydrofolate (△), 5-$CH_3$—$H_4PteGlu$ (□), CH=$H_4PteGlu_1$ (■), folic acid (▲), 5-CHO—$PteGlu_1$ (leucovorin) (◇), and 10-CHO—$H_4PteGlu_1$ (◆).

5. Results of control experiments with $CH_2$—$H_4PteGlu_1$ and $H_4PteGlu_1$ are given for reference. Below 1.0 μM, the efficiency of ternary complex formation by $H_4PteGlu_1$ was 28 ± 5% that of $CH_2$—$H_4PteGlu_1$, similar to the 37° data (Fig. 3). At the maximum concentration used, 0.5 mM folate, as a percentage of maximal ternary complex obtained with $CH_2$—$H_4PteGlu_1$, ternary complex formation decreased in the following order for the following folates: dihydrofolate (29.6%), 5-$CH_3$—$H_4PteGlu_1$ (7.5%), CH=$H_4PteGlu_1$ (3.0%), folic acid (1.6%), 5-CHO—$H_4PteGlu_1$ (leucovorin) (1.1%), and 10-CHO—$H_4PteGlu_1$ (0.9%). The effect of a 20-fold excess of dUMP (0.6 μM) at 0.5 mM folate concentration resulted in average decreases of 31.5 ± 19.6% (C/S = 1.46) in ternary complexes for these folates, which was not significantly different from the relative decreases found for TS–FdUMP binary complex controls (average C/S = 1.7). At concentrations below 10 μM, the yields of protein-bound radioactivity, greater than TS–FdUMP binary complex controls, were progressively less than for the binary complex values alone.

The specificity of dUMP for decreasing TS–FdUMP–$CH_2$—$H_4PteGlu_1$, ternary complex formation was determined by adding 20 pmol of TS and 10 pmol of $CH_2$—$H_4PteGlu_1$ to 5 pmol of [$^3$H]FdUMP pre-mixed with various amounts of CMP, AMP, UMP, dCMP, dAMP, dGMP, TMP, or TdR, with incubation for 10 min at 30° prior to charcoal isolation of protein-bound radioactivity. None of these nucleic acid precursors, up to 10 μM final concentration (a 300-fold excess over FdUMP), caused a measurable decrease in TS–[$^3$H]FdUMP–5,10-$CH_2$—$H_4PteGlu_1$ formation, with the exception of TMP. The decrease in ternary complex formation by TMP resulted in a slope of C/S values versus dTMP concentration that was 9% of the slope for dUMP under the same conditions, a result in agreement with previous data [9].

*TS activity correlations.* Decreases in TS activity, studied by $^3H_2O$ release from [5-$^3$H]dUMP, caused by a relatively high dUMP/FdUMP ratio of 40 at

low $CH_2$—$H_4PteGlu_1$ concentrations were studied at the same concentrations of TS (0.02 μM) and FdUMP (0.1 μM) used for the [$^3$H]FdUMP ligand-binding studies. Control results with 0.1 μM dUMP without FdUMP showed 85 ± 1% conversion of [$^3$H]dUMP to $^3H_2O$ at $CH_2$—$H_4PteGlu_1$ concentrations of 0.50 and 2.5 μM, and with $CH_2$—$H_4PteGlu_1$ limiting (0.1 μM), 54% consumption of $CH_2$—$H_4PteGlu_1$; with higher dUMP, 4 μM, 86% conversion occurred. Taking decreases in $^3H_2O$ release in the presence of FdUMP to represent TS–FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex formation, values of 15.1, 57.9, and 100% of maximal ternary complex formation were obtained at $CH_2$—$H_4PteGlu_1$ concentrations of 0.025, 0.1 and 0.625 μM, respectively, in good agreement with the data of Fig. 1. In the presence of a dUMP/FdUMP ratio of 40, the lower amount of ternary complex at 0.1 μM folate, 4.55% of maximal, gave a C/S value of 12.7 and a ([C/S]-1)/([dUMP]/[FdUMP]) value of 0.290. These were experimentally indistinguishable from values shown for TS–[$^3$H]FdUMP–$CH_2$—$H_4PteGlu_1$ formation (Figs. 1 and 4).

Thus, for $CH_2$—$H_4PteGlu_1$ concentrations 1.0 μM and below, the data shown in Figs 1–4 appear to represent measured decreases in enzyme activity. Because depletion of $CH_2$—$H_4PteGlu_1$ in the presence of a dUMP concentration of 4.0 μM was at least 1.6-fold greater than at low dUMP (0.1 μM), it can be suggested that most, if not all, of the difference in the Fig. 4 slopes of $CH_2$—$H_4PteGlu_1$ and $H_4PteGlu_1$ below 1.0 μM folate was due to consumption of $CH_2$—$H_4PteGlu_1$.

## DISCUSSION

Using nitrocellulose membrane separation of ligands, Lockshin and Danenberg [9] showed that the combined effects of low $CH_2$—$H_4PteGlu_1$ and high dUMP are at least multiplicative on decreasing rates of TS–[$^3$H]FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex formation, with little difference noted between bacterial (*L. casei*) and human (CCRF-CEM lymphoblast) sources of TS. The importance of this observation results from the general occurrence of high dUMP and low $CH_2$—$H_4PteGlu_n$ concentrations in human [19, 20] as well as in murine tumors receiving treatment with FUra [18]. The present report extends these studies to include a broader range of substrates, and to quantify more specifically the role of the 5,10-methylene group in the interactions of dUMP with FdUMP for binding to TS.

Competition between dUMP and FdUMP for TS–nucleotide binary complex formation showed uniform linearity between C/S (= Control values/dUMP-added Sample values) ratios and dUMP/FdUMP ratios, at various TS concentrations, using 10 min × 37° incubations. The effect of increasing inhibition of TS–FdUMP binary complex formation by dUMP with decreasing concentrations TS was helpful in excluding the possibility that trace quantities of $CH_2$—$H_4PteGlu_1$ were present. For ternary complex formation, C/S effects of competing dUMP increased markedly with TS in excess over FdUMP. This difference between binary and ternary complex formation may be related to the general finding of two binding sites for FdUMP but only one binding site for dUMP binary complexes [8, 10, 28]. In addition, positive cooperativity for ternary complex formation occurs with FdUMP, but is apparently not generally observed with dUMP, although dUMP can occupy both TS sites simultaneously in the case of pteroyltriglutamate [29], and we noted possible weak cooperativity for binary complex formation with TS in excess (see Results). Some investigators have found only one FdUMP binding site in the absence of folates [12, 13]. The basis for conflicting reports is unknown, but TS–FdUMP–$CH_2$—$H_4PteGlu_n$ ternary complexes may be found in both monomeric and dimeric forms [23].

The decreases in [$^3$H]FdUMP binding to TS by dUMP, presented as C/S values of the binary complex, presented a limit condition for the effects of variation of concentration of $H_4PteGlu_1$ (Fig. 3). Comparison of $H_4PteGlu_1$ and $CH_2$—$H_4PteGlu_1$ effects was necessary for quantitation of the relative contributions of the 5,10-methylene group to ternary complex stabilization and to decreases mediated by consumption of folate as substrate. Dimedone (5 mM) was present in our studies with $H_4PteGlu_1$ as a scavenger of formaldehyde [7], which may be a frequent contaminant [25], but studies without dimedone gave indistinguishable results.

The interference by dUMP on causing decreased ternary complex formation at $CH_2$—$H_4PteGlu_1$ concentrations below 0.5 μM, to a greater extent than predicted by simple binary complex effects alone, appeared to be largely a result of substrate conversion based on tritium-release studies of enzyme activity. The effect of $CH_2$—$H_4PteGlu_1$ at higher concentrations on diminishing dUMP interference may be interpreted on the basis of Eqn 3, which combines Eqns 5 and 14 of Lockshin and Danenberg [9]:

$$k^s_{on} = \frac{k_1 \dfrac{k_3[CH_2—H_4PteGlu_1]}{k_2 + k_3[CH_2—H_4PteGlu_1]}}{1 + [dUMP]/K_s} \qquad (3)$$

where $k^s_{on}$ is the rate of TS–FdUMP–$CH_2$—$H_4PteGlu_1$ ternary complex in the presence of dUMP, $k_1$ and $k_2$ are the FdUMP-dependent rates of TS–FdUMP binary complex formation and dissociation, $k_3$ the rate of binding of $CH_2$—$H_4PteGlu_1$ to binary complex to form ternary complex, and $K_s$ the equilibrium binding constant (about 0.4 μM, Refs 9 and 10) for dUMP binary complex formation. At high values of $k_3[CH_2—H_4PteGlu_1]$, the term $k_2$ is negligible, and overall ternary complex formation should be given by $k_1/(1 + [dUMP]/0.4\,\mu M)$. The latter expression would predict interference with rates of FdUMP ternary complex formation by dUMP concentrations over 0.4 μM, at all concentrations of $CH_2$—$H_4PteGlu_1$, as found with $H_4PteGlu_1$. However, contributing to what would appear to be equilibrium effects at concentrations of $CH_2$—$H_4PteGlu_1$ greater than 1.0 μM, above which dUMP did not appreciably interfere, likely were positive homotropic effects of first-site FdUMP ternary complex on promotion of rates of second-site

binding [8, 27], greater for $CH_2$—$H_4PteGlu_1$ than for $H_4PteGlu_1$. The sigmoidicity present in semilog plots of linear percent ternary complex formation versus log [folate] curves (see Figs 1 and 2) also supports the expected allosterism [30]. At the inflection point for $CH_2$—$H_4PteGlu_1$ (Fig. 4) at about 0.5 $\mu$M, $k_2$ may be expected to be nearly equal to the net rate of ternary complex formation, $k_3[CH_2$—$H_4PteGlu_1]/k_4$, where $k_4$ is the rate of ternary complex dissociation, so that $k_{on}$ would be expected to be relatively independent of folate concentration below this value. Published values for these constants, ($k_2$, $1.7 \times 10^4$/min, $k_3$, $1.3 \times 10^7$/M/min; and $k_4$, $1.9 \times 10^{-2}$/min) [7] give a calculated value of 0.25 $\mu$M, close to the empirical 0.5 $\mu$M value. The approximately 2-fold increase in $k_4$ for $H_4PteGlu_1$, compared to $CH_2$—$H_4PteGlu_1$ [15], therefore, may only partly account for the substantially greater difference in the interactions of these folates with dUMP (Fig. 4).

Since rates of ternary complex formation and dissociation by mammalian TS may resemble those for *L. casei* TS [9, 14], Fig. 4 may model the kinetic situation in malignant tissues. Our analysis of the efficiency of TS inhibition at given ratios of dUMP/FdUMP in fifty human cancers suggests that at least one-half of all tumors are primarily resistant to TS inhibition on the basis of low $CH_2$—$H_4PteGlu_n$ concentrations alone or associated with high dUMP levels [20], in agreement with direct assays of $CH_2$—$H_4PteGlu_n$. The effect of polyglutamation should be to increase the $K_D$ values of ternary complexes, by increased rates of complex formation as well as decreased dissociation rates [21, 22, 31–36]. One would expect *a priori* a shift in the inflection point of the curve for dUMP interference for $CH_2$—$H_4PteGlu_1$ (Fig. 4) to a lower $CH_1$—$PteGlu_n$ concentration by polyglutamation, although $K_s$ values for dUMP for the polyglutamates have not been reported. A recent report has indicated that a dUMP/FdUMP ratio of 30 results in only a 19% decrease in TS–FdUMP–$CH_2$—$H_4PteGlu_5$ ternary complex using *L. casei* TS, 0.1 $\mu$M folate, and a 5-min incubation at 30°, compared to a calculated (Fig. 4) value of 45% for the monoglutamate [35]. A possible, unconfirmed kinetic complexity is an observation of reverse sequence binding by $CH_2$—$H_4PteGlu_4$ [31].

Figure 6 shows the effect of hexaglutamation on TS-[$^3$H]FdUMP–MTX-$Glu_n$ ternary complex formation. MTX-$Glu_6$ was studied because of its availability. The rate for MTX-$Glu_6$ was approximately 50-fold faster than for MTX, similar to results reported by others [36], with the maximal yield of ternary complexes 30.2 and 19.1% of $CH_2$—$H_4PteGlu_1$ controls. The simultaneous presence of dUMP in 50-fold excess over FdUMP gave results dramatically opposite to that of $CH_2$—$H_4PteGlu_1$ and $H_4PteGlu_1$. That is, with increasing concentrations of MTX or MTX-$Glu_6$, dUMP showed increasing interference with TS–FdUMP–MTX-$Glu_n$ ternary complex formation. An implication for chemotherapy may be that high-dose MTX would more readily inhibit TS via dUMP ternary complex formation than low-dose MTX, and thus act by an additional synergistic mechanism with

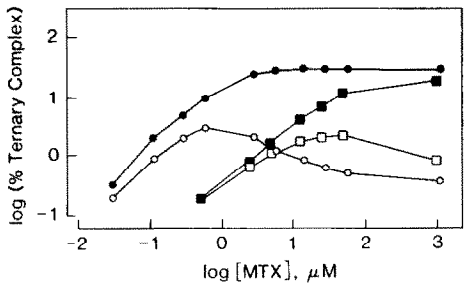


Fig. 6. Effect of hexaglutamylation on TS–[$^3$H]FdUMP–MTX-$Glu_n$ ternary complex formation and effects of dUMP. To 0.03 $\mu$M [$^3$H]FdUMP and different concentrations of the monoglutamate (MTX) (■, □) or hexaglutamate (MTX-$Glu_6$) (●, ○) of methotrexate, was added (to a final concentration of 0.10 $\mu$M) *L. casei* TS, and protein-bound radioactivity was isolated after a 10-min incubation at 30°, as in Fig. 5. The C/S effects of dUMP (open symbols) at 5 $\mu$M (dUMP/FdUMP ratio = 50) versus controls without dUMP (filled symbols), in decreasing ternary complex formation were the same for the two glutamate analogs.

FUra at the high doses, which has been suggested clinically [37].

The monoglutamate model of dUMP interaction with TS–FdUMP–folate ternary complex formation is of physiological interest for several reasons. First, it provides an unambiguous reference method for comparing different folates according to effectiveness in overcoming dUMP interference with ternary complex formation (Fig. 4). Second, it presents a probable limit, maximal target concentration of $CH_2$—$H_4PteGlu_n$ (in monoglutamate-equivalents) for overcoming dUMP interference, 1.0 $\mu$M. Third, folate flooding predictably leads to increases in the percentages of intracellular mono- and diglutamate metabolites [38–40]. Numerous successful clinical schedules use short-term infusions of leucovorin concurrently with FUra administration.

The monoglutamate studies presented here strongly suggest that intracelluar $CH_2$—$H_4PteGlu_n$ levels by TS–[$^3$H]FdUMP ligation can easily be overestimated: regardless of dUMP concentration, $H_4PteGlu_1$ is at least 20% as efficient as $CH_2$—$H_4PteGlu_1$ in forming ternary complexes resistant to denaturing conditions, in agreement with earlier estimates [6, 41]. Since estimates of $H_4PteGlu_n$ are 3- to 10-fold greater than "$CH_2$—$H_4PteGlu_n$" [21, 23, 35], much if not most of the latter may often simply represent $H_4PteGlu_n$ binding. We have used the phenomenon of interference by added dUMP as a probe of specificity for $CH_2$—$H_4PteGlu_n$ in liver biopsy specimens [42], although tissue variability in dUMP and $CH_2$—$H_4PteGlu_n$ may make this approach inconsistent. Thus, $CH_2$—$H_4PteGlu_n$ levels are likely to be even more rate-controlling for dTMP synthesis than already appreciated. Promising strategies for increasing $CH_2$—$H_4PteGlu_n$ levels after leucovorin or 5-$CH_3$—$H_4$-$PteGlu_1$ administration, such as concurrent L-serine and L-glutamate loading, are in progress [43].

The extremely poor binding of 5-CHO—

$H_4PteGlu_1$ (leucovorin) and $CH{=}H_4PteGlu_1$ into TS–FdUMP–folate ternary complexes does not exclude the additional possibilities that polyglutamate derivatives of these, following pharmacologic doses of leucovorin, can mediate increased FdUMP inhibition of TS as a basis of FUra/leucovorin synergy. Inhibition of TS is also known to occur by formation of TS–dUMP–5-CHO—$H_4PteGlu_4$ [44] and TS–dUMP–$PteGlu_3$ complexes [29].

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