Biochemical Pharmacology, Vol. 38, No. 2, pp. 335–342, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

EFFECT OF POLYGLUTAMYLATION OF 5,10-METHYLENETETRAHYDROFOLATE ON THE BINDING OF 5-FLUORO-2′-DEOXYURIDYLATE TO THYMIDYLATE SYNTHASE PURIFIED FROM A HUMAN COLON ADENOCARCINOMA XENOGRAFT

SAEED RADPARVAR, PETER J. HOUGHTON and JANET A. HOUGHTON*

Laboratories for Developmental Therapeutics, Department of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, Memphis, TN 38101, U.S.A.

(*Received* 11 *April* 1988; *accepted* 30 *June* 1988)

Abstract—CH_2-H_4PteGlu and H_4PteGlu exist in human colon adenocarcinoma xenografts predominantly in the form of polyglutamate species at concentrations of $<3\ \mu M$. The interaction of polyglutamates of [6*R*]CH_2-H_4PteGlu in the formation and stability of [6-^{3}H]FdUMP–thymidylate synthase–CH_2-H_4PteGlu$_n$ ternary complexes has therefore been examined using enzyme purified from a human colon adenocarcinoma xenograft. Dissociation of these complexes was first-order and was dependent upon the concentration of folate. [6*R*]CH_2-H_4PteGlu$_{3-6}$ (0.9 to 1.6 μM) were >200-fold and [6*R*]CH_2-H_4PteGlu$_2$ (18.2 μM) was 18-fold more effective than [6*R*]CH_2-H_4PteGlu$_1$ (335 μM) at stabilizing ternary complexes for a $T_{1/2}$ for dissociation of 100 min. Polyglutamylation of CH_2-H_4PteGlu also increased the affinity of binding of [6-^{3}H]FdUMP to thymidylate synthase as determined by Scatchard analysis at folate concentrations of 10 μM, where the K_d in the presence of [6*R*]CH_2-H_4PteGlu$_1$ was in the order of 4.0×10^{-8} M, and for [6*R*]CH_2-H_4PteGlu$_{3-5}$ was between 3.7 and 5.5×10^{-9} M. To examine whether this effect was due to differences in the rates at which [6-^{3}H]FdUMP was bound (k_{on}) or dissociated (k_{off}) from the enzyme, the apparent rate of [6-^{3}H]FdUMP binding was determined in the presence of [6*R*]CH_2H_4PteGlu$_1$, [6*R*]CH_2-H_4PteGlu$_3$ and [6*R*]CH_2-H_4PteGlu$_4$. The k_{on} values were similar and were in the range of 1.7 to $2.3 \times 10^6\ M^{-1}\ min^{-1}$ for 10 or 20 μM folate concentrations. Differences in binding affinity determined for [6*R*]CH_2-H_4PteGlu$_1$ and longer polyglutamate forms of [6*R*]CH_2-H_4PteGlu were thus due to differences in k_{off}. The V_{max} for the initial velocity of [6-^{3}H]FdUMP binding was achieved at 10 μM folate. Consequently, at concentrations of CH_2-H_4PteGlu polyglutamates present in tumors, inhibition of thymidylate synthase by FdUMP *in vivo* would be expected to be transient, based upon the concentration of [6*R*]CH_2-H_4PteGlu$_n$ required for maximal formation and stability of the covalent ternary complex. It would be advantageous for modulation of CH_2-H_4PteGlu$_n$ pools to increase the concentrations of the longer polyglutamate species ($n \geq 3$) to maximize the interaction between FdUMP, thymidylate synthase and CH_2-H_4PteGlu.

Thymidylate synthase (EC 2.1.1.45; 5,10-methylenetetrahydrofolate:dUMP, *C*-methyltransferase) catalyzes the reductive methylation of dUMP to dTMP and is an important target in the treatment of adenocarcinoma of the colon by the anticancer agent FUra.† 5-Fluorouracil is metabolized to FdUMP which forms a covalent ternary complex with thymidylate synthase in the presence of CH_2-H_4PteGlu, resulting in the inhibition of dTMP biosynthesis. The dissociation of this inhibitory complex is first-order, and dependent upon the concentration of unbound CH_2-H_4PteGlu [1, 2]. Consequently, the duration of inhibition of thymidylate synthase *in vivo* may be dependent upon the endogenous concentration of CH_2-H_4PteGlu.

Intracellularly, reduced folates exist as polyglutamate forms with glutamate residues linked through the γ-carboxyl groups [3–8]. Polyglutamates of CH_2-H_4PteGlu containing up to seven glutamate residues have increased affinity for thymidylate synthase [9–11]. However, the influence of polyglutamylation of the cofactor on the formation and stability of the ternary complex remains poorly characterized. Polyglutamylation of CH_2-H_4PteGlu may be important for increasing the velocity of binding of [6-^{3}H]FdUMP to thymidylate synthase [11, 12], for reducing the rate at which the nucleotide dissociates from the enzyme [11, 13], or for binding the folate at lower concentration [11]. In human colon adenocarcinoma xenografts, di-, tri-, tetra-, penta- and hexaglutamate forms of CH_2-H_4PteGlu have been identified [14]. Due to the importance of thymidylate synthase as a target in this disease, the

* Correspondence: Dr. J. A. Houghton, Laboratories for Developmental Therapeutics, Department of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38101.

† Abbreviations: FUra, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridylate; dUMP, deoxyuridylate; dTMP, thymidylate; [6*R*]CH_2-H_4PteGlu$_n$, the natural diastereoisomer of 5,10-methylenetetrahydrofolate where n = the number of glutamate residues; H_4PteGlu, tetrahydrofolate; PteGlu, folic acid; 5-CHO-H_4PteGlu, 5-formyltetrahydrofolate, leucovorin; DTT, dithiothreitol; BSA, bovine serum albumin; TMDG buffer, 0.1 M Tris–HCl, pH 7.5, containing 1.5 mM $MgCl_2$, 2 mM DTT and 10% glycerol; and TCA, trichloroacetic acid.

effects of these different polyglutamate species on the stability of the FdUMP–thymidylate synthase–CH_2-H_4PteGlu$_n$ complex, the affinity of binding of [6-^{3}H]FdUMP, and the rate of ternary complex formation have been examined using thymidylate synthase purified from a human colon adenocarcinoma xenograft.

METHODS

Materials. [5-^{3}H]dUMP (22 Ci/mmol) and [6-^{3}H]FdUMP (15–20 Ci/mmol) were obtained from Moravek Biochemicals, Brea, CA. Pteroylpolyglutamates containing from 2 to 7 glutamyl residues, prepared by solid phase synthesis, were purchased from American Radiochemicals Inc., St. Louis, MO. NCS tissue solubilizer and ACS and OCS liquid scintillation fluids were obtained from the Amersham Corp., Arlington Heights, IL. Folic acid (PteGlu) and all other reagents and chemicals were obtained from the Sigma Chemical Co., St. Louis, MO, or were of reagent grade.

Immune-deprivation of mice. Four-week-old CBA/CaJ mice were immune-deprived by thymectomy followed 3 weeks later by whole-body irradiation (950 rads at a dose rate of 170 rads/min) from a ^{137}Cs source, and reconstitution with 3×10^6 syngeneic bone marrow cells injected i.v. [15]. Mice received s.c. grafts of neoplastic tissue after a further 8–16 days.

Tumor line. The human colon adenocarcinoma xenograft line $HxVRC_5$ has been described previously [16–20]. Briefly, tumors were established as xenografts from a poorly differentiated primary adenocarcinoma obtained from a previously untreated patient [15, 18]. The line has maintained both biological and biochemical characteristics observed within the original human specimen [15] and has been insensitive *in vivo* to treatment with 5-fluoropyrimidines [19]. This particular tumor was selected for investigation from a series of six colorectal adenocarcinoma xenograft lines, as it has demonstrated the highest thymidylate synthase activity [16, 17]. Tumors were used for enzyme purification upon reaching ≥1 g in weight.

Purification of thymidylate synthase and assay. The purification of thymidylate synthase >4000-fold from $HxVRC_5$ human colon adenocarcinoma xenografts was performed as described previously [21]. The activity of thymidylate synthase was determined from the catalytic release of [^{3}H] from [5-^{3}H]dUMP at 37°, 3 min [21]. Standard conditions used were 10 μM [5-^{3}H]dUMP (sp. act. 2.7 Ci/mmol) and 80 μM [6*RS*]CH_2-H_4PteGlu.

Synthesis of [6*R*]CH_2-H_4PteGlu$_n$ *from PteGlu.* The natural [6*R*] isomers of CH_2-H_4PteGlu polyglutamates were prepared by enzymatic synthesis from the corresponding pteroylpolyglutamates using dihydrofolate reductase purified from methotrexate-resistant *Lactobacillus casei* and an excess of formaldehyde as previously described [21]. Their purity and concentration were determined by HPLC analysis; storage was at −80° in long glass tubes flushed with argon [21]. [6*RS*]CH_2-H_4PteGlu was prepared as previously reported [21].

Stability of the [6-^{3}*H*]*FdUMP–thymidylate synthetase–*CH_2-H_4PteGlu$_n$ *ternary complex.* Complex was formed with [6-^{3}H]FdUMP (100 nM), [6*R*]CH_2-H_4PteGlu$_1$ (10 μM), [6*R*]CH_2-H_4PteGlu$_2$ (6 μM) or [6*R*]CH_2-H_4PteGlu$_{3-6}$ (1.5 μM) and 0.5 to 1.5 ml enzyme (27–40 nM [6-^{3}H]FdUMP binding sites) in a total volume of 1.0 to 2.0 ml TMDG buffer (0.1 M Tris–HCl, 1.5 mM $MgCl_2$, 2 mM DTT and 10% glycerol, pH 7.5) at 37° over a period of 45 min and placed on ice. An equal volume of ice-cold buffer was added subsequently. To determine the stability of the ternary complex formed with [6*R*]CH_2-H_4PteGlu$_1$ in the presence of different concentrations of [6*R*]CH_2-H_4PteGlu$_1$, four reaction mixtures (total volume 800 μl) each contained 400 μl ternary complex, TMDG buffer (pH 7.5) and [6*R*]CH_2-H_4PteGlu$_1$ at concentrations of 2.5, 25, 50 or 75 μM. Non-radiolabeled FdUMP (100 μM) was also added to start the exchange reaction with [6-^{3}H]FdUMP according to the method of Lockshin and Danenberg [2]. Ternary complex and combined reagents were prewarmed separately at 37° for 3 min prior to mixing. Reaction mixtures were incubated at 37° for up to 45 min. At times 0, 15, 30 and 45 min, duplicate aliquots of 80 μl were placed on ice, and treated with 1 ml of an ice-cold 5% charcoal suspension (containing 1% BSA and 0.1% dextran in TMDG buffer, pH 7.5), to adsorb unbound FdUMP. After a further 20 min on ice, mixtures were centrifuged (12,000 *g*, 2°, 4 min), supernatant fractions were filtered, and dpm in 0.6 to 0.8 ml were determined by liquid scintillation counting. Data were analyzed by linear regression analysis to yield half-times ($T_{1/2}$) for dissociation of the ternary complex.

Alternatively, the stability of the ternary complex formed with [6*R*]CH_2-H_4-PteGlu$_2$ was determined in the presence of increasing concentrations of [6*R*]CH_2-H_4PteGlu$_2$. Four reaction mixtures (total volume 700 μl) each contained 350 μl of ternary complex, TMDG buffer, [6*R*]CH_2-H_4PteGlu$_2$ at a concentration of 1.5, 4, 8 or 15 μM, and 100 μM FdUMP. Finally, the stability of ternary complex formed with tri-, tetra-, penta- and hexaglutamates of [6*R*]CH_2-H_4PteGlu was determined in the presence of 0.38, 0.88, 1.9 or 2.9 μM [6*R*]CH_2-H_4PteGlu$_n$ using the method outlined for [6*R*]CH_2-H_4PteGlu$_1$.

Dissociation constant (K_d) *for* [6-^{3}*H*]*FdUMP in the presence of* [6*R*]CH_2-H_4PteGlu$_n$. To determine relative differences in binding affinity between [6*R*]CH_2-H_4PteGlu$_1$ and [6*R*]CH_2-H_4PteGlu$_n$, enzyme (1 to 1.5 nM [6-^{3}H]FdUMP binding sites) and [6*R*]CH_2-H_4PteGlu$_n$ (10 μM) were incubated with various concentrations of [6-^{3}H]FdUMP (0.2 to 200 nM) in a total volume of 250 μl Tris–HCl (25 mM, pH 7.4, containing 0.5% BSA, 1% β-mercaptoethanol and 10 mM sodium ascorbate), for 45 min at 37° by which time the reaction had reached equilibrium. The [6-^{3}H]FdUMP–thymidylate synthase–CH_2-H_4PteGlu$_n$ ternary complex was quantitated using TCA precipitation [22]. Briefly, duplicate reaction mixtures were treated with an equal volume of ice-cold 20% TCA to denature the protein. The mixtures were centrifuged for 5 min, 15,500 *g*, 2°, and the supernatant fractions were decanted. The precipitates were suspended subsequently in 1 ml TCA (10%), and the mixtures recentrifuged. This step was repeated twice, at which point the super-

natant fractions contained only background radioactivity. The precipitates were dissolved in 1 ml NCS at room temperature overnight, and radioactivity was determined following addition of 10 ml of OCS scintillant. Data were analyzed by the method of Munson and Rodbard [23] for a one-site binding model using the computer program "Ligand" and analyzed using an Apple IIe computer. Estimates for the association constant (K_d) were obtained using nonlinear least squares regression, where initial values were determined from the slopes of the Scatchard curves. The dissociation constant (K_d) was calculated as the inverse of K_a.

Influence of CH_2-H_4PteGlu$_n$ on the apparant rate constant for FdUMP association (k_{on}) *and dissociation* (k_{off}). Measurement of the apparent rate constant for [6-^{3}H]FdUMP association was essentially as described by Lockshin and Danenberg [2]. Reaction mixtures (1.9 ml) containing [6*R*]CH_2-H_4PteGlu$_n$ (10 or 20 μM), enzyme (4.5 nM [6-^{3}H]FdUMP binding sites), and buffer (25 mM Tris–HCl, pH 7.4, containing 1% BSA, 1% β-mercaptoethanol and 10 mM sodium ascorbate) were prewarmed at 37°; prewarmed [6-^{3}H]FdUMP (10 or 25 nM) was added to start the reaction. At intervals of up to 2 min, aliquots (300 μl) were pipetted into an equal volume of ice-cold TCA (20%), and precipitated radiolabeled ternary complex was determined as described.

Under these conditions, the apparent bimolecular rate constant (k_{on}) for [6-^{3}H]FdUMP association to thymidylate synthase was calculated from the equation

$$k_{on}t = \frac{1}{[E_0] - [\mathrm{FdUMP}_0]} \times \ln \frac{[\mathrm{FdUMP}_0\,([E_0 - [X])}{[E_0]\,([\mathrm{FdUMP}_0] - [X])}$$

where $[E_0]$ is the initial concentration of enzyme binding sites, $[\mathrm{FdUMP}_0]$ is the initial concentration of [6-^{3}H]FdUMP, and $[X]$ is the concentration of ternary complex at time t (min); k_{off} was calculated from the equation $K_d = k_{off}/k_{on}$ [2], where K_d and k_{on} had been determined experimentally.

Concentration of CH_2-H_4PteGlu$_n$ at V_{max} for [6-^{3}H]*FdUMP binding.* Reaction mixtures (100 μl), in duplicate, containing thymidylate synthase (3 nM [6-^{3}H]FdUMP binding sites), [6-^{3}H]FdUMP (25 nM), CH_2-H_4PteGlu$_n$ (1–20 μM) and 25 mM Tris–HCl, pH 7.4 (containing 1% BSA, 1% β-mercaptoethanol and 10 mM sodium ascorbate), were incubated at 37° for 2 min. Ice-cold 20% TCA (100 μl) was added to terminate the reaction, and precipitated ternary complex was determined as described. K_m values were determined by the method of Cleland [24] using computer-assisted analysis.

RESULTS

The effect of polyglutamylation of CH_2-H_4PteGlu on the stability of the [6-^{3}H]FdUMP–thymidylate synthase–CH_2-H_4PteGlu$_n$ ternary complex was examined using enzyme purified from $HxVRC_5$ human colon adenocarcinoma xenografts. Results obtained for complex formed with [6*R*]CH_2-H_4PteGlu$_4$ and subsequently reincubated with [6*R*]CH_2-H_4PteGlu$_4$ are shown in Fig. 1 in comparison to dissociation of the ternary complex formed with [6*R*]CH_2-H_4PteGlu$_1$ in the presence of various concentrations of the monoglutamate. In both instances, dissociation of complexes was first-order, and the stability was dependent upon the concentration of the folate. At the lowest concentration of [6*R*]CH_2-H_4PteGlu$_1$ used (2.5 μM), the ternary complex dissociated with a $T_{1/2}$ of 32 min; for 75 μM [6*R*]CH_2-H_4PteGlu, the $T_{1/2}$ for dissociation was increased to 53 min. However, for [6*R*]CH_2-

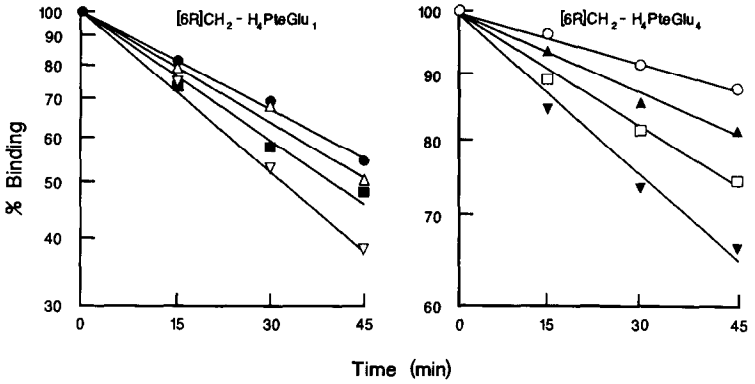

Fig. 1. Left panel: Stability of the [6-^{3}H]FdUMP–thymidylate synthase–CH_2-H_4PteGlu$_1$ complex in the presence of increasing concentrations of [6*R*]CH_2-H_4PteGlu$_1$. Reaction mixtures containing radiolabeled ternary complex (6.8 nM), a 4000-fold excess of non-radiolabeled FdUMP (100 μM) and TMDG buffer, pH 7.5, in a total volume of 800 μl were incubated at 37° for up to 45 min in the presence of (▽) 2.5, (■) 25, (△) 50 or (●) 75 μM [6*R*]CH_2-H_4PteGlu$_1$. At 15-min intervals, 80-μl aliquots, in duplicate, were placed on ice and treated with 1 ml of an ice-cold 5% charcoal suspension. The amount of ternary complex remaining was determined as described in Methods. Right panel: Stability of the [6-^{3}H]FdUMP–thymidylate synthase–CH_2-H_4PteGlu$_4$ complex in the presence of increasing concentrations of [6*R*]CH_2-H_4PteGlu$_4$. Reaction mixtures (800 μl) containing radiolabeled ternary complex (9.8 nM), FdUMP (100 μM), TMDG buffer, pH 7.5, and (▼) 0.38, (□) 0.88, (▲) 1.9 or (○) 2.9 μM [6*R*]CH_2-H_4PteGlu$_4$ were incubated at 37° for up to 45 min and processed as described to determine the amount of ternary complex remaining. All data were evaluated by linear regression analysis (left panel, $r^2 > 0.989$; right panel $r^2 > 0.963$).

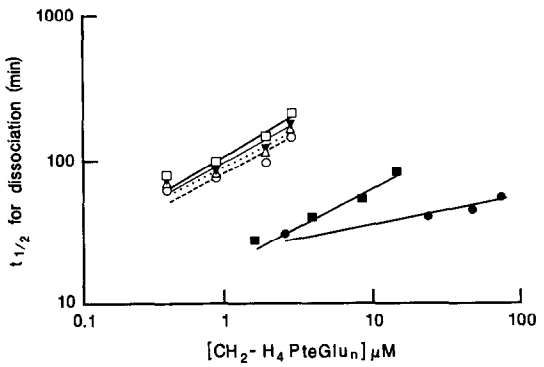

Fig. 2. Relationship between $T_{1/2}$ for dissociation and concentration of [6*R*]CH_2-H_4PteGlu$_n$. Data were derived from experiments described in the legend to Fig. 1 and were evaluated by linear regression analysis ($r^2 > 0.991$). Key: (●——●) [6*R*]CH_2-H_4PteGlu$_1$, (■——■) [6*R*]CH_2-H_4PteGlu$_2$, (△ · · · △) [6*R*]CH_2-H_4PteGlu$_3$, (□——□) [6*R*]CH_2-H_4PteGlu$_4$, (○———○) [6*R*]CH_2-H_4PteGlu$_5$, and (▼——▼) [6*R*]CH_2-H_4PteGlu$_6$.

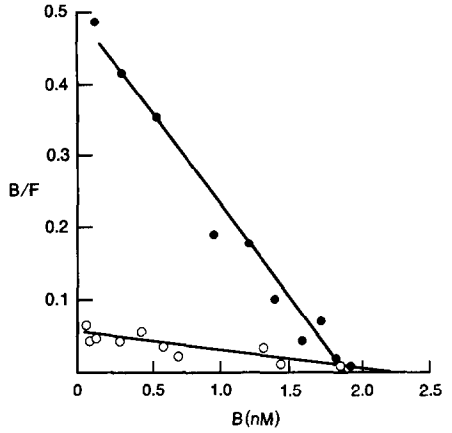

Fig. 3. Scatchard analysis for the binding of [6-^{3}H]FdUMP to thymidylate synthase in the presence of (○) [6*R*]CH_2-H_4PteGlu$_1$ or (●) [6*R*]CH_2-H_4PteGlu$_4$. Reaction mixtures containing thymidylate synthase (1 to 1.5 nM), [6*R*]CH_2-H_4PteGlu$_n$ (10 μM), [6-^{3}H]FdUMP (0.2 to 200 nM) and 25 mM Tris–HCl buffer, pH 7.4, containing 1% β-mercaptoethanol, 10 mM sodium ascorbate and 0.5% BSA in a total volume of 250 μl, were incubated at 37° for 45 min. Ternary complex formed was determined by acid precipitation as described in Methods. Data were analyzed for a one-side binding model by the method of Munson and Rodbard [23].

H_4PteGlu$_4$ at a concentration of 0.38 μM, the $T_{1/2}$ for dissociation was 73 min, and it was increased to 215 min in the presence of 2.9 μM tetraglutamate.

The relationship between the $T_{1/2}$ for dissociation and concentration of CH_2-H_4PteGlu$_n$ was determined for n = 1 to 6 (Fig. 2). The magnitude of the difference between [6*R*]CH_2-H_4PteGlu$_1$ and [6*R*]CH_2-H_4PteGlu$_4$ was similar for [6*R*]CH_2-H_4PteGlu$_3$, [6*R*]CH_2-H_4PteGlu$_5$ and [6*R*]CH_2-H_4PteGlu$_6$; [6*R*]CH_2-H_4PteGlu$_2$ was somewhat more effective at stabilizing the ternary complex in comparison to [6*R*]CH_2-H_4PteGlu$_1$, but it was inferior to the longer polyglutamate chain length forms of the cofactor. Quantitation of the data from linear regression analyses yielded concentrations of [6*R*]CH_2-H_4PteGlu$_{3-6}$ (0.9 to 1.6 μM) that were >200-fold lower than required for [6*R*]CH_2-H_4PteGlu$_1$ (335 μM) to stabilize the complex with a $T_{1/2}$ for dissociation of 100 min (Table 1); for this degree of stabilization, 18 μM [6*R*]CH_2-H_4PteGlu$_2$ was required. When ternary complex was formed with [6*R*]CH_2-H_4PteGlu$_1$ and incubated in the presence of increasing concentrations of [6*R*]CH_2-H_4PteGlu$_4$, the relationship between $T_{1/2}$ for dissociation and folate concentration was identical to data derived from formation and stabilization of the complex with [6*R*]CH_2-H_4PteGlu$_4$ alone (data not shown).

To determine the effect of polyglutamylation of CH_2-H_4PteGlu on the affinity of binding of [6-^{3}H]FdUMP to thymidylate synthase, enzyme was incubated with 10 μM [6*R*]CH_2-H_4PteGlu$_n$ and various amounts of [6-^{3}H]FdUMP at 37° for 45 min prior to measurement of bound [6-^{3}H]FdUMP using TCA precipitation. Data plotted according to the Scatchard equation are shown in Fig. 3 for [6*R*]CH_2-H_4PteGlu$_1$ and [6*R*]CH_2-H_4PteGlu$_4$, where a large difference in slopes of the lines was evident. Using computer-assisted nonlinear least squares regression analysis [23], data fitted a one-site binding model; K_d values determined for [6*R*]CH_2-H_4PteGlu$_1$, [6*R*]CH_2-H_4PteGlu$_3$, [6*R*]CH_2-H_4PteGlu$_4$ and [6*R*]CH_2-H_4PteGlu$_5$ are shown in Table 2. From two separate experiments, the K_d for [6-^{3}H]FdUMP binding in the presence of [6*R*]CH_2-H_4PteGlu$_1$ was in the order of 4×10^{-8} M. Addition of two glutamate residues decreased the binding constant by

Table 1. Concentrations of CH_2-H_4PteGlu$_n$ required to stabilize the ternary complex with a $T_{1/2}$ for dissociation of 100 min*

CH_2-H_4PteGlu$_n$ n	Concentration (μM)	Ratio $\frac{[CH_2\text{-}H_4PteGlu_1]}{[CH_2\text{-}H_4PteGlu_{2\text{-}6}]}$
1	335	1
2	18.2	18
3	1.3	268
4	0.9	372
5	1.6	216
6	1.2	291

* Calculated from linear regression analysis.

Table 2. Dissociation constants (K_d) for the binding of [6-^{3}H]FdUMP to thymidylate synthase in the presence of CH_2-H_4PteGlu$_n$*

CH_2-H_4PteGlu$_n$ n	K_d (M)	×-fold
1	4.2×10^{-8}	1
	3.8×10^{-8}	1
3	4.0×10^{-9}	10.1
	3.7×10^{-9}	10.8
4	3.8×10^{-9}	10.5
5	5.5×10^{-9}	7.3

* Reaction mixtures contained 1 to 1.5 nM enzyme, 10 μM [6*R*]CH_2-H_4PteGlu$_n$, and varied concentrations of [6-^{3}H]FdUMP (0.2 to 200 nM), as described in Methods.

Table 3. Rates of association (k_{on}) and dissociation (k_{off}) for the binding of [6-^{3}H]FdUMP to thymidylate synthase purified from $HxVRC_5$ tumors

CH_2-H_4PteGlu$_n$ n	k_{on}* (M^{-1} min^{-1})	k_{off}† (min^{-1})
1	2.0×10^6 (10)	8.0×10^{-2}
	1.9×10^6 (20)	
3	1.7×10^6 (20)	
4	2.3×10^6 (10)	8.7×10^{-3}
	2.1×10^6 (20)	

* Reaction mixtures contained 4.5 nM enzyme, 10 or 25 nM [6-^{3}H]FdUMP and 10 or 20 μM [6*R*]CH_2-H_4PteGlu$_n$, indicated in parentheses.

† Estimated from the equation $K_d = k_{off}/k_{on}$ [2] for 10 μM [6*R*]CH_2-H_4PteGlu$_n$.

10-fold. The binding affinity was also 7- to 10-fold higher for [6*R*]CH_2-H_4PteGlu$_4$ and [6*R*]CH_2-H_4PteGlu$_5$ in comparison to [6*R*]CH_2-H_4PteGlu$_1$.

To determine whether the change in K_d for the binding of [6-^{3}H]FdUMP to thymidylate synthase in the presence of [6*R*]CH_2-H_4PteGlu polyglutamates was due to differences in the rates at which FdUMP was bound (k_{on}) or dissociated (k_{off}), the apparent rate of [6-^{3}H]FdUMP binding to $HxVRC_5$ thymidylate synthase in the presence of [6*R*]CH_2-H_4PteGlu$_1$, [6*R*]CH_2-H_4PteGlu$_3$ and [6*R*]CH_2-H_4PteGlu$_4$ (10 or 20 μM) and two concentrations of [6-^{3}H]FdUMP (10 or 25 nM) was determined (Table 3). Data for [6*R*]CH_2-H_4PteGlu$_4$ (20 μM) are shown in Fig. 4. Irrespective of the number of glutamate residues added to CH_2-H_4PteGlu, the apparent k_{on} values for [6-^{3}H]FdUMP binding were similar for concentrations of both 10 and 20 μM, and were determined to be between 1.7 and 2.3×10^6 M^{-1} min^{-1}. From the equation $K_d = k_{off}/k_{on}$, the k_{off} was calculated to be 8.0×10^{-2} min^{-1} for [6*R*]CH_2-H_4PteGlu$_1$ (10 μM) and 8.7×10^{-3} min^{-1} for [6*R*]CH_2-H_4PteGlu$_4$ (10 μM; Table 3).

The effect of the concentrations of [6*R*]CH_2-H_4PteGlu$_n$ on the initial velocity of binding of [6-^{3}H]FdUMP (25 nM) to thymidylate synthase was determined for [6*R*]CH_2-H_4PteGlu$_1$, [6*R*]CH_2-H_4PteGlu$_3$ and [6*R*]CH_2-H_4PteGlu$_5$ at 37° over a period of 2 min, during which time the reaction was linear (Fig. 5). The relationship between initial velocity and folate concentration was similar among the polyglutamate species. From computer-assisted analysis, the K_m values were determined to be 3.7 μM for each polyglutamate form; at 10 μM [6*R*]CH_2-H_4PteGlu$_n$, the rate of [6-^{3}H]FdUMP binding had reached a maximum.

DISCUSSION

The existence in mammalian cells of folate species as polyglutamate forms is well established. For CH_2-H_4PteGlu, the presence of polyglutamates in L1210 cells was suggested when the covalent ternary complex isolated from these cells was found to have a higher molecular weight (1000 daltons) than the FdUMP–thymidylate synthase–CH_2-H_4PteGlu$_1$

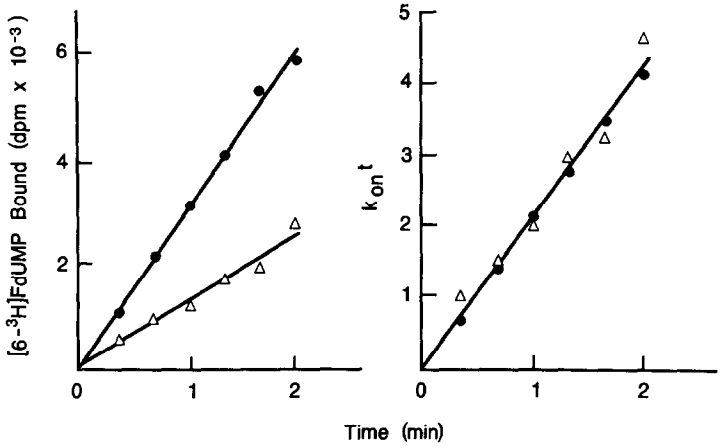

Fig. 4. Determination of the apparent k_{on} for [6-^{3}H]FdUMP binding in the presence of [6*R*]CH_2-H_4PteGlu$_4$. Reaction mixtures contained thymidylate synthase (4.5 nM), [6*R*]CH_2-H_4PteGlu$_4$ (20 μM), [6-^{3}H]FdUMP [(△) 10 nM or (●) 25 nM] and 25 mM Tris–HCl buffer, pH 7.4, containing 1% β-mercaptoethanol, 10 mM sodium ascorbate and 1% BSA, in a total volume of 1.9 ml. Incubation was at 37° for up to 2 min. At timed intervals, aliquots (300 μl) were pipetted into an equal volume of ice-cold 20% TCA, and the amount of ternary complex formed was determined as described in Methods.

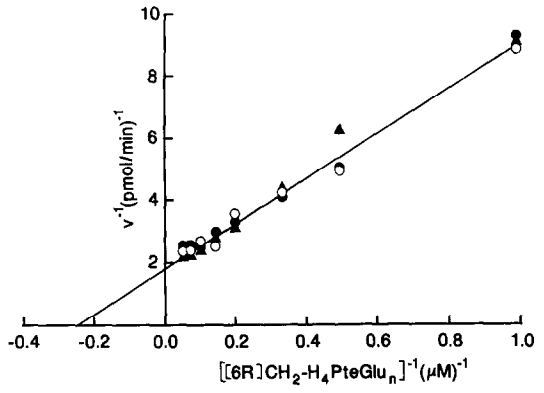

Fig. 5. Relationship between initial velocity (v) of ternary complex formation and concentration of (●) [6*R*]CH_2-H_4PteGlu$_1$, (▲) [6*R*]CH_2-H_4PteGlu$_3$ and (○) [6*R*]CH_2-H_4PteGlu$_5$. Reaction mixtures containing thymidylate synthase (3 nM), [6-^{3}H]FdUMP (25 nM), Tris–HCl buffer (25 mM, containing 1% BSA, 1% β-mercaptoethanol and 10 mM sodium ascorbate, pH 7.4), and [6*R*]CH_2-H_4PteGlu$_n$ (1–20 μM) were incubated at 37° for 2 min. Reactions were terminated and ternary complexes quantitated after acid precipitation as described in Methods.

complex when analyzed by gel electrophoresis [25]. More specifically, the predominant forms in mammalian liver and kidney have been determined to be the penta- and hexa-glutamates [26, 27], the relative distribution for liver dependent upon species. Similarly, in two human colon adenocarcinoma xenografts examined, CH_2-H_4PteGlu$_5$ constituted between 46 and 65% of the total CH_2-H_4PteGlu$_n$ pool, with CH_2-H_4PteGlu$_6$ ranging from 22 to 27% of the pool; di-, tri- and tetra-glutamates could also be detected, but in lower amounts [14]. Consequently, in attempting to understand the interaction between FdUMP, CH_2-H_4PteGlu and thymidylate synthase in human colon adenocarcinoma xenografts, it was considered important to determine the way in which the various polyglutamate species of CH_2-H_4PteGlu affected the formation and stability of the ternary complex.

The interaction of FdUMP, thymidylate synthase and [6*R*]CH_2-H_4PteGlu$_1$ has been well characterized for bacterial enzyme derived from *L. casei* [1] and mammalian thymidylate synthase using CCRF-CEM human leukemic cells as the enzyme source [2]. In both instances, an ordered mechanism of ligand binding was determined, the nucleotide binding prior to the folate, with dissociation of [6*R*]CH_2-H_4PteGlu$_1$ prior to FdUMP. Such an ordered mechanism resulted in the dependence of the stability of the complex upon the concentration of [6*R*]CH_2-H_4PteGlu$_1$, but not FdUMP. The influence of [6*R*]CH_2-H_4PteGlu$_5$ on the stability of the ternary complex has been reported for thymidylate synthase purified from MCF-7 human breast cancer cells [11], where increased stabilization was determined ($T_{1/2} \approx 187$ min) in comparison to [6*R*]CH_2-H_4PteGlu$_1$ ($T_{1/2} \approx 57$ min) at lower concentration (38 and 1 μM respectively). For the colon tumor enzyme, approximately 120 μM [6*R*]CH_2-H_4PteGlu$_1$ and 5 μM [6*R*]CH_2-H_4PteGlu$_5$ would be required to achieve similar levels of stability, data for [6*R*]CH_2-H_4PteGlu$_1$ being closer to the value determined for enzyme isolated from CCRF-CEM cells (≈180 μM; [2]). In the current study, [6*R*]CH_2-H_4PteGlu$_{3-6}$ were similar in their capacities to stabilize the ternary complex and were considerably more effective than either [6*R*]CH_2-H_4PteGlu$_2$ or [6*R*]CH_2-H_4PteGlu$_1$. This effect for [6*R*]CH_2-H_4PteGlu$_{3-6}$ was accompanied by an increased binding affinity for [6-^{3}H]FdUMP as determined by a 7- to 10-fold decrease in K_d determined by Scatchard analysis at 10 μM [6*R*]CH_2-H_4PteGlu$_n$. The differences between [6*R*]CH_2-H_4PteGlu$_1$ and [6*R*]CH_2-H_4PteGlu$_5$ were in the range reported for thymidylate synthase purified from human breast cancer cells [11]. For enzyme from this source, a 2.4-fold increase in k_{on} and a 3-fold decrease in k_{off} were responsible for the difference in K_d reported for the polyglutamate species. However, in the current study, the increasing binding affinity for [6*R*]CH_2-H_4PteGlu$_n$ appeared to be due solely to a decrease in the rates of dissociation (k_{off}). Thus, current data demonstrating that the dissociation of FdUMP was a function of the concentration of the polyglutamate species of CH_2-H_4PteGlu confirm the ordered mechanism of ligand binding as originally proposed [1, 2]. This is in contrast to suggestions by others [28] that the binding sequence observed with polyglutamates is in the reverse order from that seen with the monoglutamyl cofactor.

Concentrations of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ in cells have been estimated in the range of 3 μM or less [29, 30]. In three human colon adenocarcinoma xenografts examined, the combined pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ were determined to be from 0.5 to 2.7 μM, and for CH_2-H_4PteGlu$_n$ alone, in the order of 0.19 to 1.7 μM [14]. Ullman *et al.* [31] found in L1210 cells that the concentrations of reduced folates required for optimal cell growth (100 nM) are suboptimal for achieving maximal cytotoxicity with FdUrd (500 nM). In addition, the presence of a high proportion of intracellular folates as polyglutamates (90%) has been considered important in enhancing the antithymidylate effect of FUra in cultured cells [32]. In human colon xenografts, however, there is no correlation between the distribution of polyglutamate species and the sensitivity of tumors to FUra [14]. It is also clear that concentrations of CH_2-H_4PteGlu$_1$ required to stabilize the covalent ternary complex (>300 μM) would be considerably higher than those achievable physiologically in colon tumors, even with supplements of reduced folates such as 5-CHO-H_4PteGlu. Concentrations of CH_2-H_4PteGlu polyglutamates also appear suboptimal for forming ternary complex at the maximal rate (10 μM) or for stabilizing the complex to a level that would cause significant and prolonged inhibition of thymidylate synthase in human colon adenocarcinomas (several hours). In human colon adenocarcinoma xenografts [19], and in patients with colorectal adenocarcinoma [33, 34], responses to FUra are transient. More recently, the combination of FUra with the reduced folate 5-CHO-H_4PteGlu (leucovorin) has increased the response rates by 3- to 5-fold for this disease from data generated in randomized clinical trials [35–37],

significantly prolonging the time to disease progression [35, 37] and overall survival [37]. Although no experimental studies have been reported to date, this is presumed to be due to an increase in the pools of CH_2-H_4PteGlu that, in turn, increase the formation and stability of the FdUMP–thymidylate synthase–CH_2-H_4PteGlu ternary complex and the degree and duration of thymidylate synthase inhibition. Data from our current study indicate that the choice of a schedule for leucovorin administration should be one that increases the concentrations of the longer polyglutamate chain length forms of CH_2-H_4PteGlu to optimize the interaction between FdUMP, thymidylate synthase and CH_2-H_4PteGlu.

Acknowledgements—The authors would like to thank Dr. Jim Appleman for help with the computer-assisted analyses and for the gift of *L. casei* dihydrofolate reductase.

REFERENCES

1. Danenberg PV and Danenberg KD, Effect of 5,10-methylenetetrahydrofolate on the dissociation of 5-fluoro-2'-deoxyuridylate from thymidylate synthetase: Evidence for an ordered mechanism. *Biochemistry* **17**: 4018–4024, 1978.
2. Lockshin A and Danenberg PV, Biochemical factors affecting the tightness of 5-fluorodeoxyuridylate binding to human thymidylate synthetase. *Biochem Pharmacol* **30**: 247–257, 1981.
3. Brown JP, Davidson GE and Scott JM, The identification of the forms of folate found in the liver, kidney and intestine of the monkey and their biosynthesis from pteroylglutamate (folic acid). *Biochim Biophys Acta* **343**: 78–88, 1974.
4. Eto I and Krumdieck CL, Determination of three different pools of reduced one-carbon-substituted folates. II. Quantitation and chain-length determination of the pteroylpolyglutamates of rat liver. *Anal Biochem* **115**: 138–146, 1981.
5. Foo SK and Shane B, Regulation of folypoly-γ-glutamate synthesis in mammalian cells. *In vivo* and *in vitro* synthesis of pteroylpoly-γ-glutamates by Chinese hamster ovary cells. *J Biol Chem* **257**: 13587–13592, 1982.
6. Leslie GI and Baugh CM, The uptake of pteroyl[^{14}C]glutamic acid into rat liver and its incorporation into the natural pteroyl poly-γ-glutamates of that organ. *Biochemistry* **13**: 4957–4961, 1974.
7. McBurney MW and Whitmore GF, Characterization of a Chinese hamster cell with a temperature-sensitive mutation in folate metabolism. *Cell* **2**: 183–188, 1974.
8. Taylor RT and Hanna ML, Folate-dependent enzymes in cultured Chinese hamster cells: Folypolyglutamate synthetase and its absence in mutants auxotrophic for glycine + adenosine + thymidine. *Arch Biochem Biophys* **181**: 331–334, 1977.
9. Kisliuk RL, Gaumont Y, Lafer E, Baugh CM and Montegomery JA, Polyglutamyl derivatives of tetrahydrofolate as substrates for *Lactobacillus casei* thymidylate synthase. *Biochemistry* **20**: 929–934, 1981.
10. Dolnick BJ and Cheng Y, Human thymidylate synthetase II. Derivatives of pteroylmono- and -polyglutamates as substrates and inhibitors. *J Biol Chem* **253**: 3563–3567, 1978.
11. Allegra CJ, Chabner BA, Drake JC, Lutz R, Rodbard D and Jolivet J, Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. *J Biol Chem* **230**: 9720–9726, 1986.
12. Priest DG and Mangum M, Relative affinity of 5,10-methylenetetrahydrofolylpolyglutamates for the *Lactobacillus casei* thymidylate synthetase–5-fluorodeoxyuridylate binary complex. *Arch Biochem Biophys* **210**: 118–123, 1981.
13. Danenberg PV and Lockshin A, Tight-binding complexes of thymidylate synthetase, folate analogs, and deoxyribonucleotides. *Adv Enzyme Regul* **20**: 99–110, 1982.
14. Houghton JA, Williams LG, Radparvar S and Houghton PJ, Characterization of the pools of 5,10-methylenetetrahydrofolate and tetrahydrofolates in xenografts of human colon adenocarcinomas. *Cancer Res* **48**: 3062–3069, 1988.
15. Houghton JA and Taylor DM, Maintenance of biological and biochemical characteristics of human colorectal tumours during serial passage in immune-deprived mice. *Br J Cancer* **37**: 199–212, 1978.
16. Houghton JA, Weiss KD, Williams LG, Torrance PM and Houghton PJ, Relationship between 5-fluoro-2'-deoxyuridylate, and thymidylate synthase activity subsequent to 5-fluorouracil administration in xenografts of human colon adenocarcinomas. *Biochem Pharmacol* **35**: 1351–1358, 1986.
17. Houghton JA, Maroda SJ, Phillips JO and Houghton PJ, Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in human colorectal adenocarcinoma xenografts. *Cancer Res* **41**: 144–149, 1981.
18. Houghton JA and Taylor DM, Growth characteristics of human colorectal tumors during serial passage in immune-deprived mice. *Br J Cancer* **37**: 213–223, 1978.
19. Houghton JA and Houghton PJ, On the mechanism of cytotoxicity of fluorinated pyrimidines in four human colon adenocarcinoma xenografts maintained in immune-deprived mice. *Cancer* **45**: 1159–1167, 1980.
20. Houghton JA and Houghton PJ, Elucidation of pathways of 5-fluorouracil metabolism in xenografts of human colorectal adenocarcinoma. *Eur J Cancer Clin Oncol* **19**: 807–815, 1983.
21. Radparvar S, Houghton PJ and Houghton JA, Characteristics of thymidylate synthase purified from a human colon adenocarcinoma. *Arch Biochem Biophys* **260**: 342–350, 1988.
22. Moore MA, Ahmed F and Dunlap RB, Isolation of the covalent binary complex of 5-fluorodeoxyuridine and thymidylate synthetase by trichloroacetic acid precipitation. *Biochem Biophys Res Commun* **124**: 37–43, 1984.
23. Munson PJ and Rodbard D, Ligand: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107**: 220–239, 1980.
24. Cleland WW, Statistical analysis of enzyme kinetic data. In: *Methods in Enzymology* (Ed. Parich DL), Vol. 63, pp. 103–138. Academic Press, New York, 1979.
25. Washtien WL and Santi DV, Assay of intracellular free and macromolecular-bound metabolites of 5-fluorodeoxyuridine and 5-fluorouracil. *Cancer Res* **39**: 3397–3404, 1979.
26. Priest DG, Happel KK, Mangum M, Bednarek JM, Doig MT and Baugh CM, Tissue folypolyglutamate chain-length characterization by electrophoresis as thymidylate synthetase–fluorodeoxyuridylate ternary complexes. *Anal Biochem* **115**: 163–169, 1981.
27. Priest DG and Doig MT, Evaluation of folypolyglutamates by electrophoretic separation of fluorodeoxyuridylate–thymidylate synthase–methylene tetrahydrofolate complexes. In: *Folyl and Antifolyl Polyglutamates* (Eds. Goldman ID and Chabner BA), pp. 139–148. Plenum Publishing, New York, 1983.
28. Lu Y-Z, Aiello PD and Matthews RG, Studies on the polyglutamate specificity of thymidylate synthase from fetal pig liver. *Biochemistry* **23**: 6870–6876, 1986.

29. Moran RG, Werkheiser WC and Zakrzewski SF, Folate metabolism in mammalian cells in culture. Partial characterization of the folate derivatives present in L1210 mouse leukemia cells. *J Biol Chem* **251**: 3569–3575, 1976.
30. Rosenblatt DS, Wong PWK, Berlow S, Narisawa K and Baumgartner R, Folate distribution in cultured human cells. *J Clin Invest* **63**: 1019–1025, 1979.
31. Ullman B, Lee M, Martin DW and Santi DV, Cytotoxicity of 5-fluoro-2′-deoxyuridine: Requirement for reduced folate cofactors and antagonism by methotrexate. *Proc Natl Acad Sci USA* **75**: 980–983, 1978.
32. Yin M-B, Zakrzewski SF and Hakala MT, Relationship of cellular cofactor pools to the activity of 5-fluorouracil. *Mol Pharmacol* **32**: 190–197, 1983.
33. Carter SK and Friedman M, Integration of chemotherapy into combined modality treatment of solid tumors. II. Large bowel carcinoma. *Cancer Treat Rev* **1**: 111–129, 1974.
34. Gilbert JM, Adjuvant chemotherapy of large bowel cancer. *Cancer Treat Rev* **9**: 195–228, 1982.
35. Doroshow JH, Bertrand M, Multhauf P, Leong L, Goldberg D, Margolin K, Carr B, Akman S and Hill R, Prospective randomized trial comparing 5-FU versus (vs) 5-FU and high dose folinic acid (HDFA) for treatment of advanced colorectal cancer. *Proc Am Soc Clin Oncol* **6**: 96, 1987.
36. Petrelli N, Herrera L, Stulc J, Rustum Y and Mittelman A, A phase III study of 5-fluorouracil (5-FU) versus 5-FU + methotrexate (MTX) versus 5-FU + high dose leucovorin (CF) in metastatic colorectal adenocarcinoma. *Proc Am Soc Clin Oncol* **6**: 74, 1987.
37. Erlichman C, Fine S, Wong A and Elhakim T, A randomized trial of fluorouracil and folinic acid in patients with metastatic colorectal carcinoma. *J Clin Oncol* **6**: 469–475, 1988.