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BIOCHEMICAL FACTORS AFFECTING THE TIGHTNESS OF 5-FLUORODEOXYURIDYLATE BINDING TO HUMAN THYMIDYLATE SYNTHETASE*

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Abstract—Ligand binding studies of thymidylate synthetase (5,10-methylenetetrahydrofolate: deoxyuridylate *C*-methyltransferase, EC 2.1.1.45) isolated from CCRF-CEM human lymphoblastic leukemia cells were conducted to examine the mechanism of 5-fluorodeoxyuridylate (FdUMP) binding to the human enzyme in the presence of L-1-(+)-5,10-methylenetetrahydrofolate (5,10-CH_2H_4PteGlu) and to assess biochemical factors which could account for decreased binding of FdUMP by the enzyme in cells and tissues. Scatchard plots showed one class of binding sites for FdUMP with an apparent dissociation constant (K_D) of 3.1×10^{-11} M in the absence, and 3.5×10^{-10} M in the presence, of 80 mM potassium phosphate (P_i). The observed rate constant for FdUMP association (k_{on}^{obs}) was dependent on the 5,10-CH_2H_4PteGlu concentration and attained a maximal value of 1.7×10^8 M^{-1} min^{-1} at a concentration of *ca.* 12 μM 5,10-CH_2H_4PteGlu. Increasing the concentration of 5,10-CH_2H_4PteGlu decreased the apparent rate constant of FdUMP dissociation (k_{off}^{obs}), although FdUMP had no effect on the rate of 5,10-CH_2H_4PteGlu dissociation. These studies showed that CCRF-CEM thymidylate synthetase has an ordered mechanism of ligand binding and release, with the nucleotide binding first and dissociating last. Incubation of the enzyme–FdUMP–5,10-CH_2H_4PteGlu ternary complex with the substrate dUMP resulted in renewal of enzyme activity at about the same rate as that of FdUMP release. Nucleotides, deoxyuridine (dUrd), and polyoxyanions decreased the rate of FdUMP association but had no significant effect on the rate of FdUMP dissociation. dUMP was the most potent inhibitor of FdUMP binding found, with a binding constant determined from competition experiments of 0.36 μM compared to a Michaelis constant of 2.8 μM. The binding constant for P_i was determined similarly to be 10 mM. The effects of dUMP plus P_i on decreasing the rate of FdUMP association were additive, whereas the combined effects of dUMP and sub-optimal concentrations of 5,10-CH_2H_4PteGlu were not additive, but multiplicative or greater. The levels of dUMP, P_i and 5,10-CH_2H_4PteGlu that reportedly are present in some cells and tissues, theoretically could increase the K_D to more than 10^{-6} M. These results could account for the coexistence of substantial levels of FdUMP and unbound thymidylate synthetase found in some cells and tissues.

Despite extensive biochemical and clinical research and the use of FUra‖ as a chemotherapeutic agent, certain aspects of the mechanism of action of FUra* are not fully understood. In particular, the reasons for variability of tumor response to the drug remain obscure. In many model systems, inhibition by the metabolite FdUMP of thymidylate synthetase and, thereby, of DNA synthesis appears responsible for the effects of the drug [1, 2]. In other cases, however, metabolism of FUra to ribonucleotides and their incorporation into RNA are believed to cause cytotoxicity [3, 4]. Myers *et al.* [2] showed that the intracellular dUMP pools, which expanded in tumor-bearing mice after FUra administration, could be a a factor in decreasing the cytotoxic effect of FUra by relieving FdUMP inhibition of dTMP synthesis. Ardalan *et al.* [5], however, observed no apparent correlation between the FdUMP/dUMP ratio and the responsiveness of two murine colonic adenocarcinomas to FUra. Klubes *et al.* [6] found that the persistence of FdUMP in rodent tumors correlated best with sensitivity to FUra, although the endogenous dUMP pool was greater and expanded somewhat more in the nonresponsive solid Walker 256 carcinoma than in the responsive solid L1210 tumor. Increased thymidylate synthestase activity has also been associated with resistance to FdUrd [7, 8], a precursor of FdUMP in cells, and, in one case, a murine tumor resistant to fluoropyrimidines contained an altered thymidylate synthetase that was not inhibited by FdUMP [9]. Various enzymes involved in the catabolic and anabolic metabolism of FUra have also been implicated in tumor resist-

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‖ Abbreviations: FUra, 5-fluorouracil; FdUMP, 5-fluorodeoxyuridylate; FdUrd, 5-fluorodeoxyuridine; 5,10-CH_2H_4PteGlu, 5,10-methylenetetrahydrofolate (subscript *n* represents multiple glutamate residues); H_4PteGlu, tetrahydrofolate; dUrd, deoxyuridine and P_i, inorganic phosphate.

ance to this drug [1, 10]. No definitive variables, however, have been found, to date, which predict why some human tumors but not others are susceptible to chemotherapy with fluoropyrimidines.

Another factor limiting our knowledge about the effects of FUra on human tumors is that most investigations on the mechanism of interaction of FdUMP with thymidylate synthetase have been conducted with enzyme that was isolated from methotrexate-resistant *Lactobacillus casei* because of its ready availability. With this enzyme it was first shown that FdUMP binds to the enzyme in the presence of excess 5,10-CH_2H_4PteGlu to form a very tight-binding covalent ternary complex ($K_D = 10^{-10}$ to 10^{-12} M) [11–14]. It was also observed that the *L. casei* enzyme ternary complex can be slowly dissociated enzymatically, allowing full regeneration of enzymatic activity leading to dTMP synthesis if the ratio of dUMP/FdUMP is sufficiently high [15].

We report here a kinetic study of the interaction of CCRF-CEM human thymidylate synthetase with FdUMP. Our aims in this study were 3-fold: (1) to compare the mechanisms of FdUMP binding of the *L. casei* and human enzymes; (2) to determine the tightness of FdUMP binding with the CCRF-CEM enzyme; and (3) to investigate biochemical factors that may affect the intracellular binding of FdUMP to human thymidylate synthetase.

MATERIALS AND METHODS

Materials. L-1-(+)-CH_2H_4PteGlu [16] and FdUMP [17] were synthesized as described. [^{3}H]FdUMP (20 Ci/mmole) was purchased from Moravek Biochemicals (City of Industry, CA) and repurified as needed on small columns of DEAE-cellulose by stepwise elution with ammonium bicarbonate (pH 8.0) followed by lyophilization [18]. [5-^{3}H]dUMP (12 Ci/mmole) was also purchased from Moravek Biochemicals and lyophilized prior to use. [3′,5′,7,9-^{3}H]Folic acid (potassium salt, 29 Ci/mmole) was purchased from Amersham/Searle (Arlington Heights, IL). Other biochemicals were from the Sigma Chemical Co. (St. Louis, MO).

CCRF-CEM human lymphoblastic leukemic cells were grown in suspension [16] and provided by the Los Angeles County/University of Southern California Cell Culture Core Facility. The cells retained the morphology of human lymphoblasts. Dihydrofolate reductase purified from *L. casei* was a gift from Dr. Richard G. Moran.

L-1-(+)[^{3}H]H_4PteGlu (2 Ci/mmole) was made by enzymatic reduction of [^{3}H]folate with *L. casei* dihydrofolate reductase. The reaction was carried out in a cuvette which contained 80 μM [^{3}H]folate, 0.55 mM NADPH, 50 mM Tris–HCl, pH 7.4, 16 mM dithiothreitol, and enzyme, in a total volume of 500 μl. The reaction was allowed to proceed at 20° until there was no further decrease of absorption at 340 nm. [^{3}H]H_4PteGlu was purified on a DEAE-cellulose column (3 × 1.1 cm) using a linear gradient of 0.05 to 0.4 M ammonium acetate, pH 7.0, followed by lyophilization to dryness of fractions containing the tritiated product which was obtained in 90 per cent.

Thymidylate synthetase preparation and assay. Homogeneous enzyme was obtained from CCRF-CEM human leukemic cells and assayed spectrophotometrically as described previously [16] or by the tritium release assay of Roberts [19] modified by use of the standard reaction mixture of the spectrophotometric assay plus [5-^{3}H]dUMP (0.43 Ci/mmole) instead of unlabeled dUMP. Binding of [^{3}H]FdUMP to the enzyme in the presence of 5,10-CH_2H_4PteGlu was assayed by nitrocellulose disk filtration [16, 20] or by separation of protein-bound from unbound [^{3}H]FdUMP with a 1:5 dilution of activated charcoal containing bovine serum albumin and dextran [18]. Radioactivity was determined [16] with a counting error of less than 4 per cent for all samples. All binding studies were conducted at 37° in 50 mM Tris–HCl buffer, pH 7.4, containing 0.15 M KCl, 6 mM formaldehyde, 8 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin. The enzyme was quantitated as 1/1.7 moles of [^{3}H]FdUMP bound in the presence of saturating [^{3}H]FdUMP and 5,10-CH_2H_4PteGlu [21].

Kinetic studies. The rate of [^{3}H]FdUMP association to thymidylate synthetase in the presence of 5,10-CH_2H_4PteGlu was determined by rapid sampling of reaction mixtures containing low concentrations of enzyme (0.6 to 4 nM ligand binding sites) and [^{3}H]FdUMP (0.5 to 17 nM) in the volumes indicated below, unless otherwise noted. The reaction mixture containing all components except enzyme was equilibrated at 37°, and then 40–48 μl of prewarmed (15 sec at 37°) enzyme plus bovine serum albumin were added to start the reaction (final volume, 480 μl). Aliquots (54 μl) were removed at timed intervals and quickly pipetted into 170 μl portions of an ice-cold quench solution of 0.4 M KCl containing 0.1 mM unlabeled FdUMP. After completion of the time course, assays were filtered through nitrocellulose disks to determine the amount of protein-bound [^{3}H]FdUMP. No dissociation of [^{3}H]FdUMP from the enzyme was detected when the quenched mixtures were kept at 0° for up to 6 hr.

Dissociation of the thymidylate synthetase–FdUMP–5,10-CH_2H_4PteGlu ternary complex was also studied at 37°. The complex was preformed with either [^{3}H]FdUMP (4.4 nM enzyme binding sites, 40–50 μM 5,10-CH_2H_4PteGlu, and 19 nM [^{3}H]FdUMP) or with [^{3}H]-5,10-CH_2H_4PteGlu (2.5 nM enzyme binding sites, 130 nM [^{3}H]-5,10-CH_2H_4PteGlu, and 7.6 nM FdUMP) by incubation at 37° for 30 min. The rate of tritiated-ligand release was determined by adding a large excess of unlabeled ligand (at least 500-fold) to portions of the preformed ternary complex (thereby effectively preventing the rebinding of labeled ligand) and filtering aliquots of the reaction mixture periodically through nitrocellulose filters.

Calculation of apparent FdUMP association rate constants.* In all experiments, the concentration of the 5,10-CH_2H_4PteGlu cofactor was much higher

* Rate constants are designated as "apparent" because the interaction of FdUMP with the enzyme is not a simple bimolecular reaction (see Discussion). These constants do, however, describe the actual rates of enzyme–FdUMP–5,10-CH_2H_4PteGlu ternary complex formation and dissociation under given conditions.

(10^2 to 10^4-fold) than the concentrations of thymidylate synthetase binding sites or of [^{3}H]FdUMP. Under these conditions, the apparent bimolecular rate constant (k_{on}) of [^{3}H]FdUMP association to the enzyme was calculated from the second-order equation [22]:

$$\frac{1}{[E_0] - [\mathrm{FdUMP_0}]} \ln \frac{[\mathrm{FdUMP_0}]([E_0] - [X])}{[E_0]([\mathrm{FdUMP_0}] - [X])} = k_{on}\, t \quad (1)$$

where $[E_0]$ is the initial concentration of enzyme binding sites, $[\mathrm{FdUMP_0}]$ is the initial concentration of [^{3}H]FdUMP, and $[X]$ is the concentration of the thymidylate synthetase–[^{3}H]FdUMP–5,10-CH_2H_4PteGlu ternary complex determined at time t (in min). For [^{3}H]FdUMP concentrations substantially higher (at least 8-fold) than the concentration of enzyme binding sites, the rate equation could be simplified:

$$\ln \frac{[E_0] - [X]}{[E_0]} = -k_{on}\, t\, [\mathrm{FdUMP_0}] \quad (2)$$

with an error of less than 4 per cent for binding to the first 25 per cent of the sites.

The decrease in the rate of inhibition by a tight-binding inhibitor (present at a concentration substantially in excess of that of the enzyme) by a competing substrate can be presented, in the simplest case, by the equation [22]:

$$\ln \frac{[E_0] - [X]}{[E_0]} = -\frac{k_{on}\,[I_0]\, t}{1 + \dfrac{[S]}{K_s}} \quad (3)$$

where $[I_0]$ is the initial inhibitor concentration, $[X]$ is the enzyme–inhibitor complex (inhibited enzyme) determined at time t after addition of inhibitor, $[S]$ is the substrate concentration, and K_s is the substrate binding constant. The same relationship holds whether the decrease in enzyme activity or the progressive binding of the inhibitor to the enzyme is measured. Equation (3) is also valid for determining the rate of ligand binding for an enzyme that has multiple, equivalent binding sites, such as CCRF-CEM thymidylate synthetase (see Results), except that $[E_0]$ then represents the binding site concentration, and $[X]$ is the concentration of the binding site–inhibitor complex. For our studies with thymidylate synthetase, FdUMP was the inhibitor and dUMP was the substrate $[S]$.

When $[E_0]$ was similar to or greater than $[\mathrm{FdUMP_0}]$, the observed rate constant (k^s_{on}) in the presence of a particular $[S]$ was calculated by the second-order equation which took into account the decrease of unbound FdUMP:

$$\frac{1}{[E_0] - [\mathrm{FdUMP_0}]} \ln \frac{[\mathrm{FdUMP_0}]\,([E_0] - [X])}{[E_0]([\mathrm{FdUMP_0}] - [X])} = \frac{k_{on}\, t}{1 + \dfrac{[S]}{K_s}} = k^s_{on}\, t \quad (4)$$

where

$$k^s_{on} = \frac{k_{on}}{1 + \dfrac{[S]}{K_s}} \quad (5)$$

A graph of the left-hand term of equation (4) versus time results in a straight line of slope k^s_{on}. Plotting $1/k^s_{on}$ at constant $[E_0]$ and $[\mathrm{FdUMP_0}]$ versus $[S]$ gives a line with slope $1/K_s k_{on}$. If the value of k_{on} is known, K_s can be readily calculated. The observed rate constants for compounds that decrease the rate of FdUMP binding are designated generally as k^{obs}_{on}, or specifically, eg. $k^{P_i}_{on}$ for P_i. The k^{obs}_{on} values were determined from timed assays of limited duration, such that the combined error due to complex dissociation and to substrate depletion (where dUMP was used as a competitor of FdUMP binding) was no greater than 10 per cent.

Calculation of apparent FdUMP dissociation rate constants (k^{obs}_{off}). These were determined from the first-order dissociation of radiolabeled ligand from the preformed ternary complex [15]:

$$\ln \frac{[X]}{[X_0]} = -k^{obs}_{off}\, t \quad (6)$$

Determination of the apparent dissociation constants (K_D) *for FdUMP binding to thymidylate synthetase in the presence of* 5,10-CH_2H_4PteGlu. K_D values were determined kinetically from the ratio of the apparent rate constants of FdUMP dissociation and association at 37°. Some K_D values were obtained by equilibrium measurements at 24°, according to the Scatchard equation [23].

Slopes, intercepts, and standard errors of all lines were calculated with a computer assisted linear regression program designed by Dr. Andrew Peterson.

RESULTS

Scatchard analysis of enzyme–FdUMP binding. Enzyme was incubated with 5,10-CH_2H_4PteGlu (75 μM) and with various amounts of [^{3}H]FdUMP for 6 hr at 24°, and the binding data were graphed according to the Scatchard equation (Fig. 1). In either the absence or presence of 80 mM P_i, pH 7.4, the results showed a single class of binding sites, but the dissociation constants differed by more than an order of magnitude: the K_D was $3.1 \pm 0.3 \times 10^{-11}$M in the absence of P_i and $3.6 \pm 0.3 \times 10^{-10}$M in the presence of P_i.

Apparent rate constant for FdUMP association (k_{on}). The rate of [^{3}H]FdUMP binding to CCRF-CEM thymidylate synthetase in the presence of saturating (75 or 150 μM) 5,10-CH_2H_4PteGlu was determined to be $1.7 \pm 0.3 \times 10^8\ \mathrm{M^{-1}}$ at 37° (mean value ± standard deviation of sixteen separate determinations). Results of representative experiments are shown in Fig. 2, where the k_{on} was determined with the same enzyme binding site concentration and two different initial concentrations of FdUMP. Similar results were obtained when the initial FdUMP concentration was constant and the binding site concentration was varied. In the absence of dUMP or P_i, gradual inactivation of the enzyme usually occurred after incubation (2 min) at 37°.

[^{3}H]FdUMP binding to the enzyme in the absence of the cofactor was undetectable by nitrocellulose filtration. The effect of 5,10-CH_2H_4PteGlu concentration on the rate of [^{3}H]FdUMP binding to CCRF-CEM thymidylate synthetase is shown in Fig. 3. At

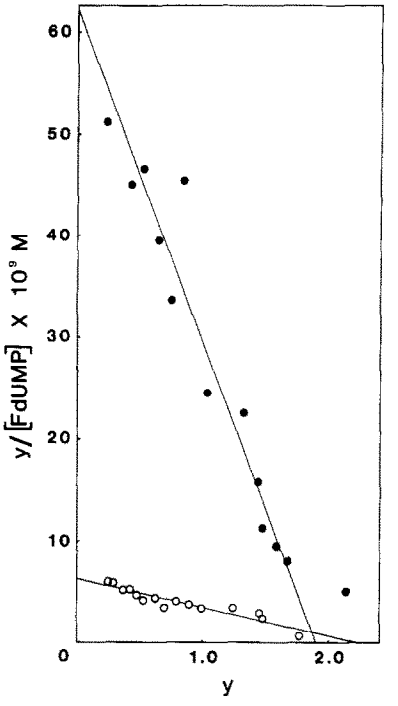


Fig. 1. Scatchard plot of [^{3}H]FdUMP binding to thymidylate synthetase in the presence (○——○) and absence (●——●) of P_i. Enzyme (0.17 nM binding sites) and 75 μM 5,10-CH_2H_4PteGlu were combined with various concentrations (0.040 to 3.8 nM) of [^{3}H]FdUMP in a total volume of 480 μl. After incubation at 24° for 6 hr, aliquots were removed to determine bound and free [^{3}H]FdUMP by the charcoal method described in Materials and Methods. The y denotes fmoles FdUMP bound per fmole enzyme.

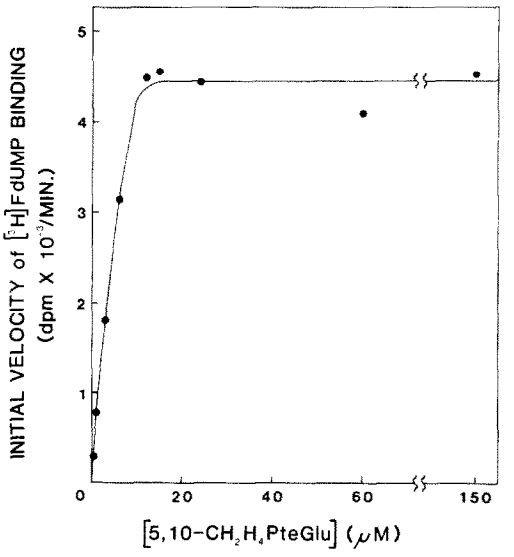


Fig. 3. Effect of the concentration of 5,10-CH_2H_4PteGlu on the initial rate of [^{3}H]FdUMP binding to thymidylate synthetase. Initial concentrations of enzyme binding sites and [^{3}H]FdUMP were 3.9 and 2.7 nM, respectively, in a total volume of 720 μl. Aliquots (90 μl) were removed and treated as described in Materials and Methods.

around 12 μM 5,10-CH_2H_4PteGlu, the binding rate reached a maximum and remained constant up to at least 150 μM cofactor. The K_m for 5,10-CH_2H_4PteGlu in the normal enzymatic reaction (with dUMP) was found to be 21 μM.

Apparent rate constants for FdUMP dissociation (k_{off}^{obs}). FdUMP dissociated from the CCRF-CEM enzyme ternary complex according to first-order kinetics, and the k_{off}^{obs} decreased when the concentration of exogenous cofactor was increased. Figure 4 shows the results of an experiment in which the rate of [^{3}H]FdUMP release at 37° was studied at concentrations of 5,10-CH_2H_4PteGlu ranging from 29 to 460 μM. The time for one-half dissociation ($T_{1/2}$) of the complex varied from 49 to 403 min as the cofactor concentration was increased. In the absence of unlabeled FdUMP, the [^{3}H]FdUMP-labeled ternary complex retained nearly 99 per cent of its original radioactivity after 4 hr. Further incubation of the complex at 37° with or without unlabeled FdUMP resulted in its irreversible breakdown. In a separate experiment, the preformed ternary complex was freed of unbound ligands by gel fitration at 4° through Sephadex G-25 prior to incubation with excess

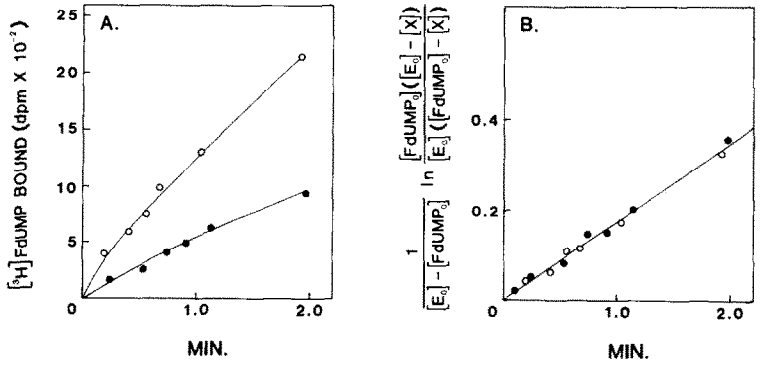


Fig. 2. Panel A: Rate [^{3}H]FdUMP binding to CCRF-CEM thymidylate synthetase. Enzyme (final concentration 2.1 nM binding sites) was added to assay mixtures containing 0.15 mM 5,10-CH_2H_4PteGlu and 0.50 nM [^{3}H]FdUMP (●——●) or 1.3 nM [^{3}H]FdUMP (○——○). Aliquots were pipetted periodically into quench solution and filtered through nitrocellulose disks as described in Materials and Methods. Panel B: Replot of these data according to equation (1).

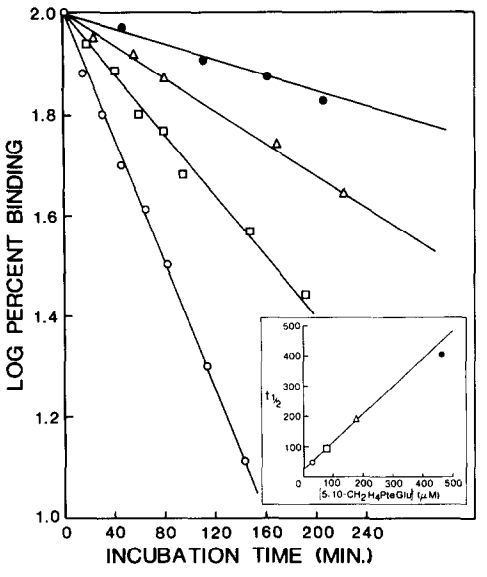


Fig. 4. Dissociation of [^{3}H]FdUMP from the thymidylate synthetase–[^{3}H]FdUMP–5,10-CH_2H_4PteGlu ternary complex in the presence of 0.2 mM FdUMP as a function of 5,10-CH_2H_4PteGlu concentration. Each reaction mixture contained complex formed from 2.9 nM binding sites and 13 nM [^{3}H]FdUMP. Portions of the preformed ternary complex were preincubated at 37° with various concentrations of 5,10-CH_2H_4PteGlu, and FdUMP was added to start the exchange with [^{3}H]FdUMP. Aliquots of the reaction mixture were filtered periodically through nitrocellulose disks. The concentrations of 5,10-CH_2H_4PteGlu in the assay mixtures were: 29 μM (○——○), 75 μM (□——□), 180 μM (△——△), and 460 μM (●——●). Insert: Replot of these data. $T_{1/2}$ is the time in min for dissociation of one-half of the [^{3}H]FdUMP from the ternary complex.

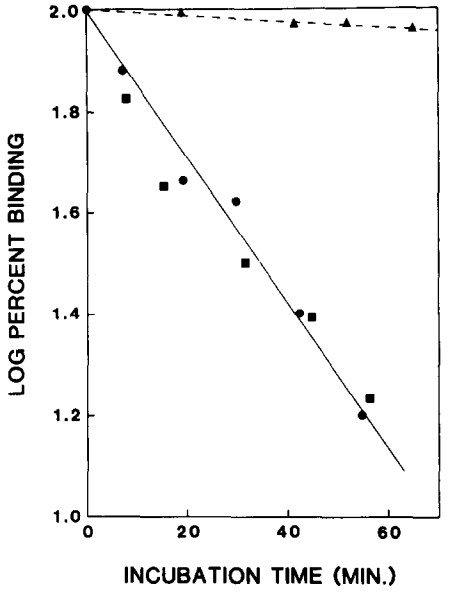


Fig. 5. Dissociation of 5,10-CH_2H_4PteGlu from the thymidylate synthetase–FdUMP–5,10-CH_2H_4PteGlu ternary complex as a function of FdUMP concentration. Each reaction mixture contained ternary complex formed from 6.0 nM enzyme binding sites and 0.32 μM 5,10-CH_2H_4PteGlu. Aliquots were filtered periodically through nitrocellulose disks. Key: 1 μM FdUMP (▲——▲); 1 μM FdUMP and 150 μM 5,10-CH_2H_4PteGlu (●——●); and 500 μM FdUMP and 150 μM 5,10-CH_2H_4PteGlu (■——■).

FdUMP at 37°. In the absence of exogenous cofactor, [^{3}H]FdUMP was released from the complex with a $T_{1/2}$ of 22 min.

An analogous experiment was conducted using [^{3}H]-5,10-CH_2H_4PteGlu as the radiolabeled ligand. As shown in Fig. 5, the $T_{1/2}$ for release of the tritiated cofactor was also 22 min and was not altered by a 500-fold increase in the concentration of exogenous FdUMP.

When the preformed ternary complex was incubated with excess (5 mM) dUMP (instead of FdUMP) at a cofactor concentration of 38 μM, the $T_{1/2}$ for recovery of enzymatic activity leading to thymidylate formation was 55 min, in substantial agreement with the value found for dissociation of [^{3}H]FdUMP from the ternary complex at that concentration of cofactor. These results demonstrate that enzymatic FdUMP release is accompanied by reactivation of the human enzyme.

Calculation of K_D *from kinetic constants.* At a concentration of 75 μM 5,10-CH_2H_4PteGlu, the k_{on} was 1.7 ± 0.3 × 10^8 M^{-1} min^{-1} and the k_{off}^{obs} was 7.5 ± 0.5 × 10^{-3} min^{-1} ($T_{1/2}$ = 93 min) (Fig. 4). The ratio of k_{off}^{obs}/k_{on} yields a K_D value of 4.4 × 10^{-11} M, which is in excellent agreement with the K_D determined by equilibrium measurements (Fig. 1).

Effects of nucleotides and other compounds on the kinetic constants of FdUMP binding. Table 1 summarizes the effects of various compounds on the rate of [^{3}H]FdUMP association to CCRF-CEM thymidylate synthetase in the presence of 150 μM cofactor. As would be expected, dUMP was by far the most effective inhibitor of FdUMP binding, followed by dTMP, UMP, and dCMP. At higher concentrations, dUrd also was inhibitory, as were P_i and arsenate at even higher concentrations. None of these compounds, however, had any significant effect on the

Table 1. Effects of Nucleotides and other compounds on the rate of [^{3}H]FdUMP binding to thymidylate synthetase*

Address	k_{on}^{obs} (M^{-1} min^{-1} × 10^{-8})
None	1.8 (k_{on})
dUMP (2.5 μM)	0.31
dUMP (25 μM)	0.044
dTMP (25 μM)	0.23
dCMP (25 μM)	1.5
UMP (25 μM)	1.3
UdR (250 μM)	0.83
P_i (20 mM)	0.57
P_i (100 mM)	0.17
Arsenate (20 mM)	0.90
Arsenate (100 mM)	0.23

* Enzyme (final concentration 2.1 nM binding sites) was added to a reaction mixture containing 1.3 mM [^{3}H]FdUMP, 150 μM 5,10-CH_2H_4PteGlu, and the compounds listed, in a total volume of 1.2 ml. Aliquots (90 μl) were removed periodically, quenched, and filtered as described in Materials and Methods. The apparent bimolecular rate constant observed in the presence of the substance indicated is denoted by k_{on}^{obs}.

rate of dissociation of the ternary complex (Table 2).

To investigate further the effect of dUMP on the rate of [^{3}H]FdUMP binding, k^s_{on} values were determined for the binding of [^{3}H]FdUMP to thymidylate synthetase in the presence of saturating amounts of cofactor and various amounts of dUMP. A plot of $1/k^s_{on}$ versus the dUMP concentration was linear (Fig. 6), as predicted for substrate competition with the ligand at the enzyme binding sites (equation 5). Calculation of K_s (equation 5) from this slope gave a value of 0.36 μM. This value does *not* correspond to the K_m of dUMP for the normal enzymatic reaction, which was determined to be 2.8 ± 0.2 μM by the tritium release assay.

P_i also behaved as a competitive inhibitor of FdUMP binding (Fig. 7) with a binding constant (K_p) of 10 mM. According to these determinations,

Table 2. Effects of nucleotides and other compounds on the time for one-half dissociation of [^{3}H]FdUMP from the thymidylate synthetase–[^{3}H]FdUMP–5,10-CH_2H_4PteGlu ternary complex*

Addition	$T_{1/2}$ (min)
None	21.5
FdUMP (1 mM)	20.9
dUMP (1 mM)	21.9
dTMP (1 mM)	24.7
dCMP (1 mM)	24.1
UMP (1 mM)	20.9
P_i (0.1 M)	22.6
Arsenate (0.1 M)	21.0
5,10-CH_2H_4PteGlu (0.15 mM)	607

* The preformed ternary complex was separated from unbound ligands by Sephadex G-25 gel filtration at 4° and then incubated with the compounds listed. Each reaction mixture contained complex formed from 1.5 nM binding sites. Aliquots were filtered periodically through nitrocellulose disks.

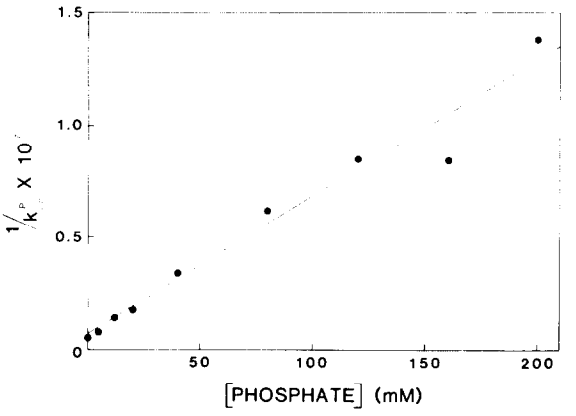


Fig. 7. Effect of the concentration of P_i on the rate of [^{3}H]FdUMP binding to thymidylate synthetase. Each reaction mixture contained 13 nM [^{3}H]FdUMP, 75 μM 5,10-CH_2H_4PteGlu and concentration of potassium phosphate, pH 7.4, from 0 to 200 mM. Enzyme (final binding site concentration of 1.2 nM) was added to start the reaction. Aliquots were removed periodically and treated as described in Materials and Methods. The values of the apparent second-order rate constants of FdUMP association in the presence of P_i (k^p_{on}) were calculated from equation 4.

80 mM P_i should increase the apparent K_D to 4.0 × 10^{-10}M. This value compares very well with the K_D of 3.5 × 10^{-10}M obtained from the Scatchard plot (Fig. 1) for the ternary complex in the presence of 80 mM P_i.

Effect of combined inhibition on the rate of FdUMP binding. dUMP, P_i and 5,10-CH_2H_4PteGlu$_n$ are normally present in tissues in concentrations (see Discussion) which would be expected to lower the rate of FdUMP binding to the enzyme and thereby increase the effective K_D of the ternary complex. It was of interest, therefore, to attempt to determine the combined effects of these substances on the

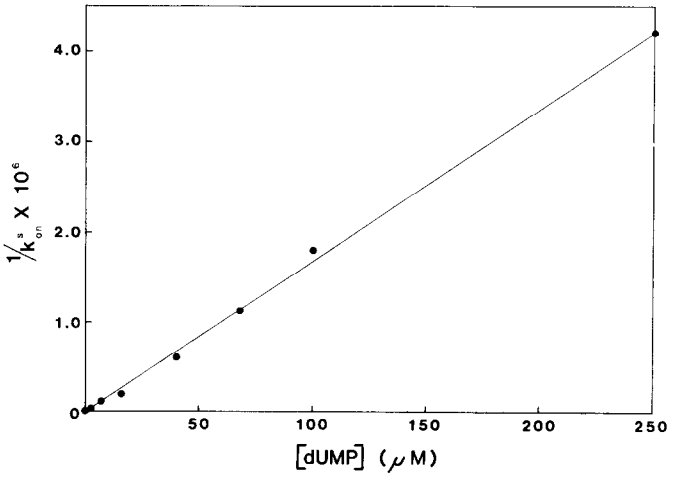


Fig. 6. Effect of the concentration of dUMP on the rate of [^{3}H]FdUMP binding to thymidylate synthetase. Each reaction mixture contained 17 nM [^{3}H]FdUMP, 75 μM 5,10-CH_2H_4PteGlu and concentrations of dUMP that varied from 0 to 250 μM. Enzyme (final binding site concentrations of 2.1 nM) was added in a total volume of 480 μl to start the reaction. Aliquots were removed periodically and treated as described in Materials and Methods. The k^s_{on} values were calculated from equation 4.

interaction of FdUMP with thymidylate synthetase.

The effects of dUMP plus P_i on the rate of [^{3}H]FdUMP binding to the enzyme were additive (Table 3), i.e.

$$\frac{k^{on}}{k_{on}^{s+p}} = \frac{k_{on}}{k_{on}^{s}} + \frac{k_{on}}{k_{on}^{p}} - 1 \qquad (7)$$

where k_{on}^{s+p} is the apparent second-order rate constant of FdUMP association to the enzyme binding sites in the presence of specific concentrations of dUMP and P_i. Equation (7) was derived as follows. From equation (5), by analogy:

$$k_{on}^{s} = \frac{k_{on}}{1 + \frac{[S]}{K_s}}, \text{ or } \frac{k_{on}}{k_{on}^{s}} = 1 + \frac{[S]}{K_s}$$

and, similarly

$$k_{on}^{p} = \frac{k_{on}}{1 + \frac{[P]}{K_p}}, \text{ or } \frac{k_{on}}{k_{on}^{p}} = 1 + \frac{[P]}{K_p} \qquad (8)$$

and, if the decrease in k_{on} by dUMP and P_i is additive, then it follows from equation 5 that:

$$\frac{k_{on}}{k_{on}^{s+p}} = 1 + \frac{[S]}{K_s} + \frac{[P]}{K_p}$$

which by substitution from equations (5) and (8) becomes equation 7.

The combined rate of decrease of [^{3}H]FdUMP binding to the enzyme in the presence of dUMP and a lower than optimal concentration of 5,10-CH_2H_4PteGlu was not additive, but multiplicative or greater (Table 4) with respect to the individual rate decreases. The apparent rate constant for FdUMP binding resulting from this multiplicative effect can be described by the equation:

$$\frac{(k_{on})}{(k_{on}^{s})}\frac{(k_{on})}{(k_{on}^{c})} = \frac{k_{on}}{k_{on}^{sc}} \qquad (10)$$

where k_{on}^{c} is the observed bimolecular rate constant at a specific concentration of 5,10-CH_2H_4PteGlu, and k_{on}^{sc} is the rate constant at the same concentration of 5,10-CH_2H_4PteGlu and a specific concentration of dUMP. Calculation according to equation (10) of the kinetic data obtained in these experiments indicated that the ratio k_{on}/k_{on}^{sc}, which quantitates the relative decrease in the rate constant of FdUMP binding brought about by a combination of dUMP and sub-optimal cofactor concentrations, was at least as large as that predicted for a multiplicative effect (Table 4). A similar effect was observed with P_i plus a sub-optimal concentration of cofactor (not shown).

DISCUSSION

The ligand binding studies reported in this paper provide information about the mechanism of FdUMP binding to human TSase and quantitate some biochemical factors that may have a considerable effect on the inactivation by FdUMP of thymidylate synthetase *in vivo*.

Although FdUMP and 5,10-CH_2H_4PteGlu form a covalently bonded complex with CCRF-CEM thymidylate synthetase [16], in the present study both ligands were dissociable, and the human enzyme regained the capacity to synthesize dTMP if excess dUMP was present. This reactivation is similar to that observed for *L. casei* thymidylate synthetase [15] and analogous to the effect of substrate on reversing inhibition of dihydrofolate reductase caused by the tightly bound, non-covalent inhibitor methotrexate [24, 25].

Increasing the concentrations of the 5,10-CH_2H_4PteGlu cofactor retarded FdUMP dissociation from the enzyme–FdUMP–5,10-CH_2H_4PteGlu ternary complex (Fig. 4), whereas FdUMP had no apparent effect on the dissociation of the cofactor (Fig. 5). These observations are in accord with the proposed ordered mechanism of ligand release and binding that had been found for the *L. casei* enzyme, whereby the cofactor must dissociate prior to FdUMP release [15]. By the principle of microscopic reversibility, FdUMP must bind first followed by the folate cofactor. A simplified diagram for ternary complex formation and dissociation is presented in Scheme 1.

Table 3. Effect of dUMP plus P_i on the rate of [^{3}H]FdUMP binding to thymidylate synthetase*

	Additions	Enzyme binding sites (nM)	FdUMP (nM)	Observed rate constants of FdUMP binding (M^{-1} min^{-1} × 10^{-8})	$\frac{k_{on}}{k_{on}^{s}} + \frac{k_{on}}{k_{on}^{p}} - 1$†	$\frac{k_{on}}{k_{on}^{s+p}}$
Expt. 1						
(a)	None	1.0	8.2	1.92 ±0.16 (k_{on}^{s})		
(b)	dUMP (4 μM)	1.0	8.2	0.131 ± 0.007 (k_{on}^{s})		
(c)	P_i (80 mM)	1.0	8.2	0.155 ± 0.013 k_{on}^{p})	26.1	
(d)	dUMP (4 μM) + P_i (80 mM)	1.0	8.2	0.078 ± 0.005 (k_{on}^{s+p})		24.6
Expt. 2						
(a)	None	1.1	7.8	1.85 ± 0.10 (k_{on})		
(b)	dUMP (2 μM)	1.1	7.8	0.197 ± 0.020 (k_{on}^{s})		
(c)	P_i (40 mM)	1.1	7.8	0.267 ± 0.012 (k_{on}^{p})	15.3	
(d)	dUMP 2 μM) + P_i (40 mM)	1.1	7.8	0.116 ± 0.014 (k_{on}^{s+p})		15.9

* Enzyme was added to start the reaction in mixtures which contained 75 μM 5,10-CH_2H_4PteGlu in a total volume of 480 μl. Aliquots were removed periodically, quenched, and filtered as described in Materials and Methods. The rate constants are the mean values ± S.E. of duplicate assays; k_{on}, k_{on}^{s}, k_{on}^{p} and k_{on}^{s+p} are defined in the text.

† From equation (7).

Table 4. Effect of the concentration of 5,10-CH_2H_4PteGlu on the inhibition of [^{3}H]FdUMP binding to thymidylate synthetase by dUMP*

	Enzyme binding sites (nM)	FdUMP (nM)	dUMP (μM)	5,10-CH_2H_4PteGlu (μM)	Observed rate constants of FdUMP binding (M^{-1} min^{-1} × 10^{-8})	$\frac{(k_{on})^2}{(k^s_{on})(k^c_{on})}$†	$\frac{(k_{on})}{(k^{sc}_{on})}$†
Expt. 1							
(a)	1.2	7.0	0	75	1.82 ± 0.51 (k_{on})		
(b)	1.2	7.0	0	4	1.08 ± 0.26 (k^c_{on})		
(c)	1.2	7.0	2.0	75	0.247 ± 0.021 (k^s_{on})	12.4	
(d)	1.2	7.0	2.0	4	0.0864 ± 0.0075 (k^{sc}_{on})		21.1
Expt. 2							
(a)	1.0	9.3	0	75	2.03 ± 0.12 (k_{on})		
(b)	1.0	9.3	0	3	1.21 ± 0.03 (k^c_{on})		
(c)	1.0	9.3	1.0	75	0.484 ± 0.021 (k^s_{on})	7.0	
(d)	1.0	9.3	1.0	3	0.194 ± 0.007 (k^{sc}_{on})		10.5

* Experiments were conducted as described in the legend to Table 3. The rate constants are the means ± S.E. of duplicate assays; k_{on}, k^c_{on}, k^s_{on} and k^{sc}_{on} are defined in the text.
† From equation (10).

$$E + FdUMP \underset{k_2}{\overset{k_1}{\rightleftarrows}} E\text{–}FdUMP \underset{k_4}{\overset{k_3[5,10\text{-}CH_2H_4PteGlu]}{\rightleftarrows}} E\text{–}FdUMP\text{–}5,10\text{-}CH_2H_4PteGlu$$

Scheme 1

It would be predicted from this proposed mechanism of action that a linear relation exists between $1/k^{obs}_{off}$ and the cofactor concentration [15]:

$$1/k^{obs}_{off} = \frac{1}{k_3} + \frac{k_3}{k_4 k_2}[C] \qquad (11)$$

where [C] is the concentration of 5,10-CH_2H_4PteGlu. Such is the case for the CCRF-CEM enzyme as shown in the insert of Fig. 4 ($T_{1/2} = \ln 2/k^{obs}_{off}$). The $T_{1/2}$ value for the vertical intercept (zero cofactor concentration) is 22 min, identical to that found for the $T_{1/2}$ of [^{3}H]FdUMP dissociation when exogenous cofactor was first removed by Sephadex G-25 filtration and to that found for the $T_{1/2}$ of [^{3}H]-5,10-CH_2H_4PteGlu dissociation from the ternary complex.

There appears to be little difference in the k_{on} of FdUMP binding for the CCRF-CEM and *L. casei* enzymes ($k_{on} = 2.0 \times 10^8\ M^{-1}\ min^{-1}$ for the *L. casei* enzyme [15], or $1.2 \times 10^8\ M^{-1}\ min^{-1}$ for enzyme binding sites). However, the effect of exogeneous cofactor on the rate of FdUMP dissociation is substantially different for the bacterial and the human enzymes. The equation for the straight line in Fig. 4 (insert) is described by the empirical equation:

$$T_{1/2} = (0.95) \times [5,10\text{-}CH_2H_4PteGlu\ (\mu M)] + 22\ min \qquad (12)$$

whereas the equation for the analogous line for the *L. casei* enzyme [15] is:

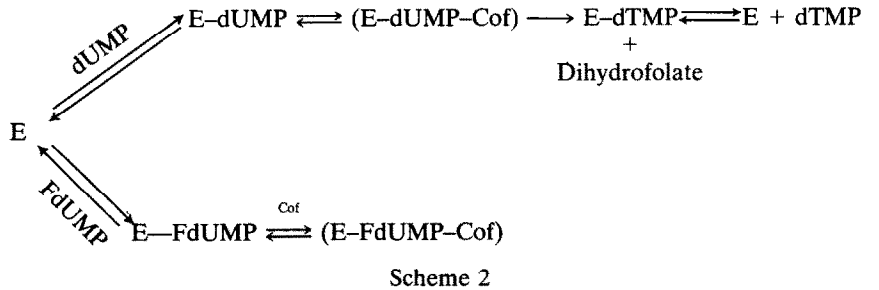
$$T_{1/2} = (0.27) \times [5,10\text{-}CH_2H_4PteGlu\ (\mu M)] + 37\ min \qquad (13)$$

From these equations, it can be seen that the effect of higher cofactor concentrations on stabilizing FdUMP binding is considerably greater with the human enzyme. At very low cofactor concentrations, FdUMP dissociates more rapidly from the human enzyme complex, but above *ca.* 22 μM cofactor the reverse is true.*

For both enzymes the K_D of FdUMP binding to thymidylate synthetase varies as the concentration of cofactor is varied. Low concentrations of cofactor decrease the apparent k^{obs}_{on}, and the amount of cofactor affects k^{obs}_{off} at concentrations at least as great as 460 μM.

By analogy with the mechanism of ternary complex formation and dissociation, the binding of substrates and release of products when dUMP instead of FdUMP interacts with the enzyme should proceed in the same order (Scheme 2).

* Widely differing dissociation rates are also observed for ternary complexes formed with FdUMP and various folate derivatives for the two enzymes (A. Lockshin and P. V. Danenberg, unpublished data).



Scheme 2

Such an ordered mechanism has been confirmed by classical kinetic studies for the *L. casei* enzyme [26] as well as for thymidylate synthetase obtained from human leukemic blast cells [27].

Kinetic enalysis of ternary complex formation with the CCRF-CEM enzyme is simplified by the apparent equivalency of the enzyme binding sites (Fig. 1). The K_D for the human enzyme in the presence of a "high" (*ca.* 0.1 mM) concentration of cofactor was about the same as that observed for *L. casei* thymidylate synthetase [11, 13, 14]. Murinson *et al.* [13] reported K_D values differing by 14-fold for the two sites of the *L. casei* enzyme, but they used the racemic mixture of (±)-5,10-CH_2H_4PteGlu rather than the L(+)-isomer in their binding studies. (−)-5,10-CH_2H_4PteGlu, an inhibitor of the *L. casei* enzyme [28], induces formation of a non-covalent complex of FdUMP to the enzyme which has a higher K_D [29] than the complex formed with the active cofactor.

Although several compounds were observed to inhibit the rate of FdUMP binding to the CCRF-CEM enzyme, none except 5,10-CH_2H_4PteGlu had any significant effect on the k_{off}^{obs}. Therefore, the increase of the K_D in each case would be proportional to $1/k_{on}^{obs}$. For most of these compounds, however, the intracellular concentrations were probably not sufficient to affect significantly the tightness of FdUMP binding *in vivo*.

The compound tested that interfered most with FdUMP binding was the competing substrate dUMP [2, 30]. Myers *et al.* [2] considered the binding constant K_s (equations 3 and 4) to be the Michaelis constant for dUMP in the enzymatic reaction, whereas we have determined its value to be 8-fold lower than the K_m of dUMP for the CCRF-CEM enzyme, and thus a substantially better inhibitor of FdUMP binding than would be expected if K_s equalled K_m. Because dUMP and FdUMP compete for formation of an enzyme–nucleotide binary complex, the K_s value may be equivalent to the dissociation constant of the enzyme–dUMP binary complex. Values ranging from 0.4 to 7.0 μM have been found for the *L. casei* enzyme–dUMP binary complex [29, 31, 32] as compared to the value of 0.36 μM that we obtained for K_s for CCRF-CEM thymidylate synthetase. Intracellular levels of dUMP vary considerably in different cells and tissues but may attain contrations well in excess of 2 mM after treatment with FUra [2, 5, 6, 18, 33]. It would be expected, therefore, that the effective K_D of FdUMP binding to thymidylate synthetase would be substantially higher *in vivo* for proliferative tissues than the K_D obtained *in vitro* by the usual ligand-binding assays conducted in the absence of dUMP.

The levels of intracellular P_i have been estimated to be 95 mM [34]. As shown both by the Scatchard plots and by the kinetic studies, P_i increased the K_D of FdUMP binding to thymidylate synthetase and also appeared to compete directly with FdUMP for the nucleotide binding sites of the enzyme. Similar conclusions were suggested by equilibrium dialysis experiments of Galivan *et al.* [29] with the *L. casei* enzyme, who found that 50 mM P_i increased the K_D of the enzyme–dUMP and the enzyme–FdUMP binary complexes several-fold. Lewis *et al.* [35] demonstrated that P_i and other polyoxyanions inhibited the rate of alkylation of the enzyme active-site sulfhydryl group by methyl methanethiosulfonate, but they inferred from their experiments that the polyoxyanions bound in close proximity to, but not directly in, the nucleotide binding site of the enzyme.

The degree to which sub-optimal 5,10-CH_2H_4PteGlu concentrations decrease the k_{on}^{obs} can be estimated from the simplified mechanism of action presented in Schemes 1 and 2, which show that ternary complex formation proceeds through two consecutive bimolecular reactions [15]. From Scheme 1, the equation for ternary complex formation can be written as:

$$k_{on} = k_1 \frac{k_3[C]}{k_2 + k_3[C]} \qquad (14)$$

Inhibition of enzyme–FdUMP binary complex formation (e.g. by dUMP) results in a smaller k_1. When $k_3[C]$ is much larger than k_2, the rate of ternary complex formation depends only on k_1. However, as the magnitude of k_2 approaches $k_3[C]$ due to decreased cofactor concentration, the overall rate will depend on the product of k_1 and $(k_3[C]/k_2 + k_3[C])$. The data indicate that the combination of dUMP or P_i plus small amounts of cofactor resulted in inhibition of k_{on} somewhat greater than predicted from equation (14), indicating that the mechanism of ternary complex association is probably more complicated than that represented in Schemes 1 and 2 [15, 36].

We have calculated what the K_D values would be, based on these data and the theoretical considerations presented in this paper, for FdUMP binding to CCRF-CEM thymidylate synthetase in the presence of various concentrations of dUMP, P_i and 5,10-CH_2H_4PteGlu. Rate constants for FdUMP binding were calculated according to equations (7) and (10), using binding constants of 0.36 μM and 10 mM for dUMP and P_i, respectively, and data for sub-optimal concentrations of cofactor from Fig. 3. Inhibition with dUMP and/or P_i in the presence of low cofactor concentrations was taken to be multiplicative in these calculations. The rate constants for FdUMP dissociation of specific cofactor concentrations were taken from the insert of Fig. 4 and equation (12). These calculations (Table 5) illustrate the profound effect on the K_D of FdUMP binding by these interfering factors.

From the above considerations, it is apparent that the extent to which thymidylate synthetase is inactivated by FdUMP *in vivo* depends principally on the concentrations of dUMP, FdUMP, enzyme, and 5,10-CH_2H_4PteGlu. Although the amounts of the first three of these constituents in cells can be determined readily [13, 18, 37], it is much more difficult to quantitate the actual concentration of 5,10-CH_2H_4PteGlu$_n$ available for interaction with thymidylate synthetase. The concentration of H_4PteGlu$_n$ plus 5,10-CH_2H_4PteGlu$_n$ in cells has been estimated to be 3 μM or less [38, 39]. However, 5,10-CH_2H_4PteGlu$_n$ serves as a substrate for other important enzymes. It is a precursor of reduced folate cofactors necessary for purine synthesis as well as of 1-carbon units transferred to several metabolites from *S*-adenosylmethionine. Moreover, H_4PteGlu$_n$

Table 5. Apparent rate and dissociation constants calculated for FdUMP binding to CCRF-CEM thymidylate synthetase in the presence of various concentrations of 5,10-CH_2H_4PteGlu, dUMP, and P_i*

5,10-CH_2H_4PteGlu (μM)	dUMP (μM)	P_i (mM)	k_{off}^{obs} ($min^{-1} \times 10^3$)	k_{on}^{obs} ($M^{-1}\,10^3$)	K_D (M)
75	0	0	7.4	1.7×10^8 (k_{on})	4.4×10^{-11}
3.0	0	0	28	6.9×10^7	4.0×10^{-10}
1.0	0	0	30	2.9×10^7	1.0×10^{-9}
75	200	0	7.4	3.1×10^5	2.4×10^{-8}
3.0	200	0	28	1.3×10^5	2.2×10^{-7}
1.0	200	0	30	5.3×10^4	5.7×10^{-7}
75	0	95	7.4	1.6×10^7	4.6×10^{-10}
3.0	0	95	28	6.5×10^6	4.3×10^{-9}
1.0	0	95	30	2.7×10^6	1.1×10^{-8}
3.0	20	95	28	1.1×10^6	2.6×10^{-8}
3.0	200	95	28	1.2×10^5	2.3×10^{-7}
3.0	2000	95	28	1.2×10^4	2.3×10^{-6}
1.0	20	95	30	4.4×10^5	6.8×10^{-8}
1.0	200	95	30	5.2×10^4	5.8×10^{-7}
1.0	2000	95	30	5.2×10^3	5.8×10^{-6}

* Apparent rate constants for FdUMP dissociation and binding were calculated as described in the text.

and 5,10-CH_2H_4PteGlu$_n$ complex with folate-binding proteins [40, 41] found in cells. Such a protein obtained from leukemic cells binds 5,10-CH_2H_4PteGlu with an affinity sufficient to inhibit thymidylate synthetase *in vitro* [41]. These combined factors indicate that the concentration of 5,10-CH_2H_4PteGlu$_n$ available for thymidylate synthetase may be quite low, perhaps 1 μM or even less.

The theoretical calculations of dissociation constants for FdUMP from the ternary complex given in Table 5 demonstrate that FdUMP does *not* behave as a stoichiometric inhibitor of thymidylate synthetase in the presence of appreciable levels of dUMP and low cofactor concentrations. Depending on these circumstances, it can be readily calculated that a substantial proportion of thymidylate synthetase would be expected to remain unbound at an enzyme concentration of *ca.* 10^{-8}M* in the presence of a 10-fold or even greater excess of FdUMP. Moreover, if a large dUMP pool is already present within cells as FUra is being metabolized to FdUMP, it would theoretically require hours or even days for binding of FdUMP to the enzyme to reach a maximum level.

It is unlikely, of course, that these calculations based on experiments with purified enzyme fully describe the interactions of thymidylate synthetase and FdUMP that take place in tissue. Washtien and Santi [42] observed that the [^{3}H]FdUMP ternary complex in intact rat hepatoma cells dissociated 3.1 times more slowly than in the cytosol obtained from disrupted cells, indicating an apparent stabilization of FdUMP binding by whole cells. If similar decreases in the k_{off}^{obs} took place in human tumor cells, the K_D values would be about 3-fold lower than those calculated in Table 5. In addition, the affinities of human thymidylate synthetases in tumors and normal proliferative tissues for FdUMP and 5,10-CH_2H_4PteGlu$_n$ may be different from the values obtained to date with enzymes isolated from other tissues. It should be noted that Jackson *et al.* [43] found that the responses of cultured mammalian cells to methotrexate were significantly influenced by the dissociation constants of the respective dihydrofolate reductase–methotrexate complexes. The interaction of other intracellular folates with thymidylate synthetase and FdUMP [11, 12, 29, 44] or dUMP [29, 32] to form ternary complexes of varying stability may also affect covalent FdUMP binding and net enzyme activity to a degree not readily predictable.

A pertinent question is how well cells can function and divide if the effective cofactor concentration is low and thymidylate synthetase is partially inhibited by FdUMP. Within cells, the cofactor exists primarily in the form of polyglutamyl conjugates. Studies with thymidylate synthetase obtained from human leukemic blast cells showed that the K_m of 5,10-CH_2H_4PteGlu$_5$ is substantially lower than the K_m of 5,10-CH_2H_4PteGlu (2.2 μM compared to 31 μM), and that the V_{max} is two times higher with the conjugated cofactor [45]. The effect of polyglutamyl derivatives of 5,10-CH_2H_4PteGlu on the tightness of FdUMP binding to thymidylate synthetase has not been reported. Ullman *et al.* [46], however, have shown that mouse leukemia L1210 cells grown in a low-folate medium with FdUrd form less of the thymidylate synthetase–FdUMP–5,10-CH_2H_4PteGlu ternary complex than do cells supplied with ample folinic acid and that the concentration of reduced folates necessary for optimal cell growth was lower than that required for maximal cytoxicity with FdUrd. These results suggest that dTMP synthesis may be adequate for cell growth and division even at low effective cofactor concentrations which contribute to reduced binding of FdUMP to thymidylate synthetase.

* In human colon carcinoma samples, the amounts of thymidylate synthetase were found to range from 2 to 40 pmoles per g tissue, or *ca.* 0.2 to 4×10^{-8}M enzyme, for most tumors. In some cases, the amount of enzyme was so low as to be undetectable by the methods used [18] (C. P. Spears, A. H. Shahinian and C. Heidelberger, personal communication).

Additional evidence suggests that most of the thymidylate synthetase must be inhibited for tumor growth to cease. Baskin *et al.* [7] found that only an 8-fold increase in the specific activity of thymidylate synthetase conferred a 2000-fold greater resistance to FdUrd by a mutant mouse neuroblastoma cell line. Houghton and Houghton [47] have reported studies showing that some human colorectal tumor xenografts implanted in nude mice did not respond to FUra treatment even though thymidylate synthetase was 90 per cent inhibited, as measured by [^{3}H]dUrd incorporation into DNA. In these xenografts, there was no correlation between incorporation of FUrd into RNA and growth inhibition, and thus it appeared unlikely that cytotoxicity was due to aberrant RNA metabolism. Gastrointestinal toxicity caused by FUra in mice, however, could be correlated with incorporation of fluorinated pyrimidines into RNA [48]. These observations have led to suggestions that for some tumours, at least, shifting the ratio of FUra metabolism from RNA incorporation to FdUMP production for inhibition of thymidylate synthetase might improve the therapeutic index with respect to gastrointestinal toxicity [48].

Our results indicate that the effectiveness of FdUMP as a stoichiometric inhibitor of human thymidylate synthetase may be severely limited by several intracellular biochemical factors. Inhibition of thymidylate synthetase by FdUMP might be enhanced by increasing the amount of intracellular cofactor, presumably by supplementation with folates in the diet, as has been suggested [15, 49], and/or by inhibiting *de novo* biosynthesis of dUMP. Further studies on the interaction of folates and folate analogues with the enzyme and FdUMP may suggest additional approaches.

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