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The Effect of Derivatives of Folic Acid on the Fluorodeoxyuridylate–Thymidylate Synthetase Covalent Complex in Human Colon Xenografts*

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Abstract—*This study was designed to examine the endogenous concentrations of 5,10-methylenetetrahydrofolate (CH_2FH_4) in human colorectal adenocarcinoma xenografts, and to determine the ability of other folate derivatives to increase the formation of the ternary covalent complex between CH_2FH_4, [6-^{3}H]-5-fluorodeoxyuridylate (FdUMP) and thymidylate synthetase (TS, EC 2.1.1.45). Levels of CH_2FH_4 were determined by measuring the release of [^{3}H]$_2$O from [5-^{3}H]-dUMP using TS from* Lactobacillus casei. *The reaction was linear from 1.9×10^{-13} to 2.4×10^{-11} mol of CH_2FH_4 assayed. Concentrations of CH_2FH_4 were low, ranging from 66 to 233 nM in cell water. Tetrahydrofolate (FH_4) and dihydrofolate (FH_2) increased complex formation, while 5-formyltetrahydrofolate (5-$CHOFH_4$) and 5-methyltetrahydrofolate (5-CH_3FH_4) decreased the covalent binding of* [6-^{3}H]-FdUMP in vitro. *Administration of FH_4 or FH_2 to tumor-bearing mice reduced subsequent formation of the covalent complex* in vitro. *Since 5-CH_3FH_4 is a major derivative of folate in mammalian tissues, its effect on the covalent binding of* [6-^{3}H]-FdUMP *was examined further; even in the presence of homocysteine and cyanocobalamin (B_{12}), the formation of the covalent complex was not increased. The fate of* [5-$^{14}CH_3$]-FH_4 *was subsequently examined* in vivo. *In tumors at* 1 hr *after injection, 72% of the radiolabel remained as* [5-$^{14}CH_3$]-FH_4, *while 17% had been converted to* [^{14}C]-*methionine or incorporated into protein. By contrast, however, the incorporation of radiolabel into the protein fraction of liver was almost 30-fold greater at this time. At* 4 hr, *radioactivity in tumors* (dpm/g) *and in the fraction associated with* [5-$^{14}CH_3$]-FH_4 *was decreased by over 60%, while metabolism was increased by only 13%. No polyglutamate forms of* [5-$^{14}CH_3$]-FH_4 *were detected in tumors at* 4 hr *after treatment.*

INTRODUCTION

THE METABOLISM of 5-fluorouracil (FUra) and its RNA- and DNA-directed mechanisms of cytotoxicity have received considerable attention; this cytotoxicity appears to depend upon the characteristics of the cells studied and the experimental conditions employed [1–3]. Consequently, we have attempted to study the effects of 5-fluorinated pyrimidines under conditions that may be more applicable to man. A series of six human colorectal xenografts, which simulate some of the heterogeneity observed clincially in this disease, have been grown as solid tumors in immune-deprived mice. Factors relating to the cytotoxicity of fluoropyrimidines have subsequently been studied under *in vivo* conditions.

Responsiveness of tumors to FUra did not correlate with the intracellular concentrations of drug achieved, levels of free FdUMP or incorporation of the drug into RNA [4]. The concentrations of unbound FdUMP achieved in tumors 1 hr after administration of FUra to tumor-bearing mice greatly exceeded the number of FdUMP binding sites on TS [5], irrespective of tumor sensitivity. Further analysis *in vitro* demonstrated that only 40–50% of the available binding sites were occupied by

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covalently bound [6-^{3}H]-FdUMP in the presence of endogenous cofactor in non-responsive tumors. To achieve maximal formation of the covalent ternary complex and complete inhibition of TS, it was necessary to add CH_2FH_4 to tumor cytosols; however, in two 5-fluoropyrimidine-sensitive lines, this was achieved without the addition of CH_2FH_4.

These data suggested that in colorectal xenografts, insensitivity to 5-fluoropyrimidines may be related to the failure to form a stable ternary complex due to low endogenous concentrations of CH_2FH_4. Currently, we have determined the endogenous concentrations of CH_2FH_4 in 6 tumor lines and have attempted to stimulate the covalent binding of [6-^{3}H]-FdUMP by the use of various folate derivatives.

MATERIALS AND METHODS

Chemicals

[5-^{14}C]-Methyltetrahydrofolic acid (58 mCi/mmol), [5-^{3}H]-dUMP (9.6 Ci/mmol) and NCS tissue solubilizer were obtained from the Radiochemical Center, Amersham, England, while [6-^{3}H]-FdUMP (18–20 Ci/mmol) was purchased from Moravek Biochemicals, City of Industry, CA. 5-Formyltetrahydrofolate (leucovorin) was obtained from Lederle Laboratories, Wayne, NJ, formaldehyde from Fisher Scientific, Pittsburgh, PA, HPLC grade methanol from Burdick and Jackson Laboratories, Muskegon, MI, and tetrabutylammonium phosphate (Pic-A) from Waters Associates Inc., Milford, MA. Thymidylate synthetase purified from methotrexate-resistant *Lactobacillus casei* was purchased from the New England Enzyme Center, Boston, MA. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO unless otherwise specified.

Tumor lines

The 6 lines of human colorectal adenocarcinomas have been described previously [5, 6]. Tumor pieces were implanted s.c. into female CBA/CaJ mice that had been immune-deprived by the procedure of thymectomy and lethal whole-body irradiation (850 rads; 4-MeV Varian linear accelerator), followed by i.v. injection of syngeneic bone marrow (2.5×10^6 cells). Four discrete tumors of the same line were maintained s.c. in each mouse. In these experiments, tumors weighing between 300 and 500 mg were used for the biochemical analyses.

Assay of CH_2FH_4

Tumor-bearing mice were anesthetized using ether. Tumors were rapidly excised and immersed in liquid nitrogen within 10 sec, and were subsequently ground to a fine powder under liquid nitrogen in a mortar. Endogenous CH_2FH_4 was extracted from approximately 200 mg of powder by boiling for 3 min in 50 mM glycine–HCl buffer, pH 9.0, containing 100 mM $MgCl_2$, 143 mM 2-mercaptoethanol and 10 mM ascorbic acid. After centrifugation, 0.5 ml of the supernatant fraction was incubated with 1 IU of 5′-nucleotidase for 30 min at 37°C to remove dUMP, and was subsequently boiled for a further 5 min to terminate the reaction. Of the extract, 25 μl was assayed for the extent of conversion of [5-^{3}H]-dUMP (4.5 μM) to dTMP by *L. casei* TS, using the method of Roberts [7]. For the standard curve, CH_2FH_4 was prepared from formaldehyde and tetrahydrofolic acid (FH_4) by the method of Moran *et al.* [8]. The protein content of extracted pellets was estimated by the method of Lowry *et al.* [9]. The volume of intracellular water was determined using established methods by evaluation of the difference between the dry weight and the [^{3}H]-inulin space of tumors.

Determination of covalently bound [6-^{3}H]-*FdUMP*

In cytosols prepared from 25% homogenates of $HxVRC_5$ tumors, the extent of [6-^{3}H]-FdUMP (54 nM) bound covalently to TS, either in the presence of endogenous cofactor or after the addition of excess CH_2FH_4, was determined by the method of Moran *et al.* [8]. Tumor was rapidly excised and homogenized at 2°C in Tris–HCl (0.01 M, pH 7.4) containing 0.25 M sucrose and 12 mM mercaptoethanol, using a Potter Elvjheim vessel fitted with a motor-driven Teflon pestle. The binding assay was carried out using 105,000 *g* supernatants as previously described [5]. Unbound [6-^{3}H]-FdUMP was separated from the covalent ternary complex by adsorption on activated charcoal coated with bovine serum albumin and dextran. The extent of covalent complex formation was determined in the presence of various folate derivatives. In these experiments FH_4, 5-CH_3FH_4 or 5-$CHOFH_4$, at concentrations of 41 μM, or 25 μM FH_2 were used in the reaction. In studies that utilized homocysteine, B_{12} and 5-CH_3FH_4, either alone or in combination, concentrations of 50 μM were used. The covalent ternary complex formed between FdUMP, TS and CH_2FH_4 is stable to conditions of denaturation, while non-covalent complexes formed with analogs such as 10-CH_3FH_4 are not [10]. In the current study, the covalent nature of the complex was characterized by acid-precipitation.

Separation of folic acid derivatives by HPLC

Mice bearing $HxVRC_5$ tumors were injected i.p. with 0.343 μCi of [5-$^{14}CH_3$]-FH_4 per gram. Tumors were excised at 1 and 4 hr, and liver at 1 hr, after treatment. Tissues were subsequently ground to a fine powder under liquid nitrogen, as described. Derivatives of folic acid for separation by HPLC were extracted by boiling powders for 5 min in 0.9% saline containing 10 mM ascorbic acid and 143 mM 2-mercaptoethanol [11]. For additional HPLC analysis, a sample of the 4-hr tumor extract was incubated at 37°C, pH 4.7, with a crude preparation of hog kidney γ-glutamylcarboxypeptidase [11] prepared by the method of Bird *et al.* [12]; thus polyglutamylfolates present in the sample are converted to monoglutamate forms. 5,10-Methenyltetrahydrofolate ($CHFH_4$) was prepared from 5-$CHOFH_4$ by the method of Rabinowitz [13]. Derivatives of folic acid (FA) in tumor extracts and standard solutions of $CHFH_4$, 5-$CHOFH_4$, FA, 5-CH_3FH_4, FH_4, FH_2 and *N*-(*p*-aminobenzoyl)-L-glutamic acid (PABG) were separated using a Partisil-10 ODS-3 column (Whatman, Inc., Clifton, NJ) with a Beckman Model 110A pump. Separation was accomplished at a flow rate of 1 ml/min using 26% methanol in water containing 0.005 M Pic-A and 97 mM 2-mercaptoethanol as the solvent. Radioactivity from tumor and liver samples was collected in 1-min fractions and counted; the u.v. absorbance was determined at 285 nm. From day to day there was some variability in the rate at which compounds were eluted from the column, although the order of elution was constant. The separation of standard compounds were thus determined at the beginning of each day, and a typical separation is shown in Fig. 3. Radioactivity associated with the protein fraction was also determined after solubilizing extracted tissue pellets in NCS.

RESULTS

The relationship between the concentration of CH_2FH_4 and the release of ^3H_2O from [5-^{3}H]-dUMP by TS is shown in Fig. 1. The reaction was linear from 1.9×10^{-13} to 2.4×10^{-11} mol of CH_2FH_4 assayed. 5,10-Methylenetetrahydrofolate was stable during the initial extraction (>90%) and also to incubation with 5'-nucleotidase, while >99.7% of the dUMP present was removed. The concentrations of CH_2FH_4 determined in the cell water of 6 lines of human colorectal adenocarcinoma xenografts are shown in Table 1. The levels ranged from 773 fmol/mg protein (66 nM) in $HxELC_2$ tumors to 2520 fmol/mg protein (233 nM) in line $HxHC_1$.

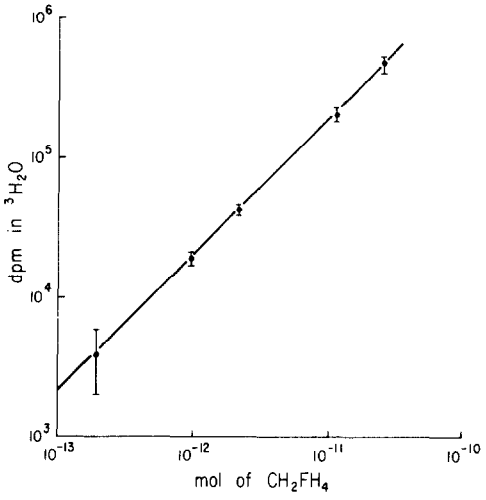

Fig. 1. Assay of CH_2FH_4. The number of moles of CH_2FH_4 was determined from the release of [^{3}H] from [5-^{3}H]-dUMP. Each point represents the mean ± 1 S.D. of 3 determinations.

Precursors of CH_2FH_4 were utilized in attempts to increase formation of the covalent complex in $HxVRC_5$ tumors. Using the [6-^{3}H]-FdUMP ligand binding assay, [6-^{3}H]-FdUMP bound covalently to TS in the presence of endogenous cofactor was expressed as 100%; the addition of CH_2FH_4 increased the covalent binding of [6-^{3}H]-FdUMP by 53%, FH_4 by 64% and FH_2 by 16%. Addition of 5-CH_3FH_4 or 5-$CHOFH_4$ decreased the formation of covalent complex by 12 and 37% respectively.

Mice bearing $HxVRC_5$ tumors were subsequently injected i.p. with either 20 mg of FH_4 or 25 mg of FH_2 per kg. At various times for up to 1 hr after treatment, tumors were excised and cytosols prepared. The amount of covalently bound [6-^{3}H]-FdUMP was then determined, both with and or without the addition of exogenous CH_2FH_4 (54 μM, Fig. 2). In the presence of endogenous cofactor, dpm associated with the ternary complex was decreased during the first 10 min, and remained below control values for up to 50 min after injection. Even after the addition of excess CH_2FH_4 to cytosols, the covalent binding of [6-^{3}H]-FdUMP was decreased in tumor cytosols prepared 10 min after injection. Complex formation returned to control values by 40 min after the administration of FH_4, but remained depressed for 1 hr after pretreatment with FH_2.

The ability of 5-CH_3FH_4 to stimulate covalent binding *in vitro* was examined further. Covalent binding of [6-^{3}H]-FdUMP to TS in the presence of 5-CH_3FH_4, with the addition of B_{12} or homocysteine, failed to increase the formation of the ternary complex (Table 3).

Table 1. Intracellular concentrations of CH_2FH_4 in tumors

Tumor line	fmol CH_2FH_4/mg protein	Concentration in cell water (nM)
HxBR	830	77
$HxAC_4$	1570	145
$HxHC_1$	2520	233
$HxGC_3$	1620	150
$HxVRC_5$	1570	158
$HxELC_2$	773	66

Table 2. The effect of folate derivatives on the formation of covalent complex in $HxVRC_5$ tumors in vitro

Folate (41 μM)	[6^3-H]-FdUMP binding (%)*
Endogenous	100 ± 2
CH_2FH_4	153 ± 1
FH_4	164 ± 12
FH_2†	116 ± 5
5-CH_3FH_4	88 ± 15
5-$CHOFH_4$	63 ± 1

*Results represent the mean ±1 S.D. of 3 determinations.
†25 μM.

Distribution of the radiolabel in tumor and liver at 1 and 4 hr after injection of [5-$^{14}CH_3$]-FH_4 is shown in Fig. 3 and Table 4. In tumor at 1 hr, 10% of the radiolabel was detected in the insoluble protein fraction; 72% of the radioactivity co-chromatographed with 5-CH_3FH_4; and 7% with methionine. At 4 hr, the total dpm/g

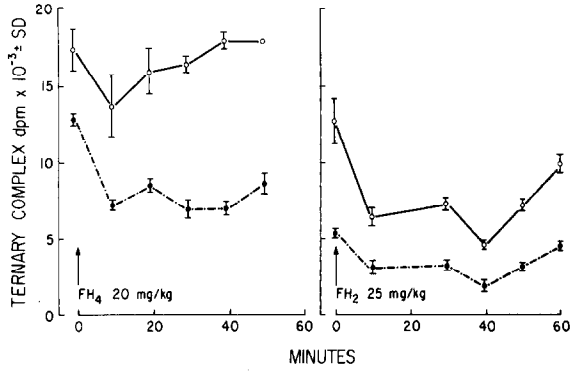

Fig. 2. The effect of pretreatment of tumor-bearing mice with FH_4 (20 mg/kg) or FH_2 (25 mg/kg) on the formation of covalent complex in vitro *in the presence of excess [6-^{3}H]-FdUMP. Tumor cytosols from line $HxVRC_5$ were assayed in the presence of endogenous cofactor (●——●) or after the addition of excess CH_2FH_4 (○——○). Each point was derived from the mean ±1 S.D. of 3 determinations.*

and the radioactivity associated with 5-CH_3FH_4 were decreased by 61 and 65% respectively; incorporation into the protein fraction was increased by 4%; and radiolabelled methionine was increased by 9%. No change in distribution of the radiolabel was obtained in the 4-hr sample from tumor after pretreatment with hog kidney conjugase. The 1–11% of dpm unaccounted for in tumor samples were considered to lie within the range of experimental error. In liver at 1 hr after treatment, 83% of the radiolabel had been incorporated into the

Table 3. The effects of homocysteine and cyanocobalamin (B_{12}) combined with 5-CH_3FH_4 on the covalent binding of [6-^{3}H]-FdUMP in cytosols from $HxVRC_5$ tumors

Addition to tumor cytosols*	[6-^{3}H]-FdUMP binding (%)†
[Endogenous CH_2FH_4]	100 ± 9
Excess CH_2FH_4	275 ± 19
5-CH_3FH_4	83 ± 2
B_{12}	77 ± 16
B_{12} + 5-CH_3FH_4	64 ± 1
Homocysteine	101 ± 8
Homocysteine + 5-CH_3FH_4	86 ± 3
Homocysteine + B_{12}	72 ± 3
Homocysteine + B_{12} + 5-CH_3FH_4	64 ± 3

*Compounds were added at a concentration of 50 μM.
†Results were obtained from the mean ±1 S.D. of 3 determinations.

Table 4. Distribution of the radiolabel in $HxVRC_5$ tumors and liver after treatment of mice with '[5-^{14}C]-methyltetrahydrofolate

Time (hr)	Tissue	Dpm/g	Percentage of total radioactivity at 1 hr			
			Insoluble fraction (Protein)	Soluble fraction 5-CH_3FH_4	Methionine	UD*
1	Tumor	344,733	10	72	7	11
4	Tumor	134,785	14	7	16	1
1	Liver	1,237,280	83	16	1	0

*UD = unidentified.

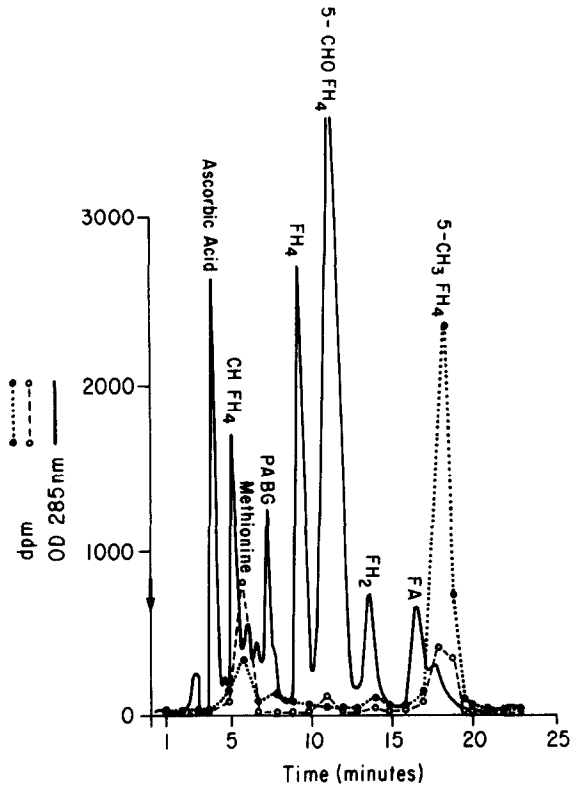

Fig. 3. The separation of folate derivatives by HPLC; (——) *elution of standard compounds. Distribution of the radiolabel was determined in HxVRC5 tumors at* 1 (●·····●) *and* 4 (○— — —○) hr *after injection of* [5-^{14}C]-*methyltetrahydrofolate. Radioactivity was collected in* 1-*min fractions.*

protein fraction, demonstrating more rapid metabolism in comparison to that obtained in tumors. A much smaller percentage (16%) was detected as unchanged [5-$^{14}CH_3$]-FH_4, while only 1% was detected as [^{14}C]-methionine.

DISCUSSION

The intracellular concentrations of CH_2FH_4 were low, ranging from 66 to 233 nM in 6 colorectal xenografts. These values are not only lower than the cofactor concentrations reported in cultured L1210 cells (3–6 μM; [11, 14]), but are at least 9- to 133-fold lower than the K_m for CH_2FH_4 (21–31 μM; [15, 16] or its pentaglutamate form (2.2 μM; [15]) obtained in studies utilizing human TS. Although it has been demonstrated that other reduced folates can stimulate the enzyme-catalyzed release of [^{3}H] from the 5-position of [5-^{3}H]-dUMP [15], if such a reaction had occurred in the current study it would lead to an overestimate of the CH_2FH_4 concentration determined. It has been demonstrated that under conditions of low cofactor concentration, the covalent ternary complex formed between CH_2FH_4, FdUMP and TS is unstable [16, 17]; in the absence of cofactor, dissociation of the complex is rapid ($t_{\frac{1}{2}}$ = 22–36 min). The rate of dissociation was independent of the external concentrations of either dUMP or FdUMP, but was reduced by increasing the concentration of CH_2FH_4. The requirement of 5-$CHOFH_4$ for the optimal growth of L1210 cells (100 nM) was reported to be lower than the requirement to achieve maximal cytotoxicity of FdUrd (> 500 nM; [18]). These data suggested that by increasing the concentration of CH_2FH_4 necessary to stabilize the covalent complex, it might be possible to enhance the cytotoxicity of fluoropyrimidines in human colon xenografts. Potential precursors of CH_2FH_4 were subsequently tested for their ability to enhance the covalent binding of [6-^{3}H]-FdUMP to TS *in vitro*. The pathways of interconversion are shown in Fig. 4. The addition of FH_4 to cytosols increased covalent complex formation. Conversion of FH_4 to CH_2FH_4 may have occurred either by the activity of serine hydroxymethyltransferase (EC 2.1.2.1), or non-enzymically by the interaction of FH_4 with formaldehyde [14, 19], which may be a minor product formed by oxidation of sarcosine or dimethylglycine in liver [20]. Both FH_2 [15] and 5-CH_3FH_4 [10] are non-competitive inhibitors of TS with regard to CH_2FH_4, while 5-$CHOFH_4$ is mutually exclusive with the non-competitive inhibitor, methotrexate [15]. Intracellular folates have been shown to form ternary complexes of varying stability with FdUMP and TS [10, 21–23] through the formation of a non-covalent bond [10, 24], although they appear not to bind FdUMP as effectively as does CH_2FH_4 [21]. The

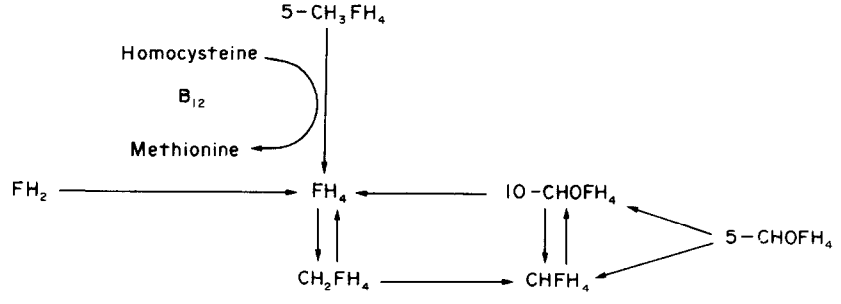

Fig. 4. Pathways of folate metabolism.

addition of 5-CHOFH$_4$ caused a greater decrease in the covalent binding of [6-^{3}H]-FdUMP than was observed with 5-CH$_3$FH$_4$, which may reflect the stronger interaction of 5-CHOFH$_4$ with the TS–FdUMP binary complex [24]. The 16% increase in covalent complex formation that was observed with FH$_2$ suggests that some conversion of FH$_2$ to FH$_4$ by dihydrofolate reductase (EC 1.5.1.4) had taken place. It is of interest that addition of CH$_2$FH$_4$ caused a greater increase in [6-^{3}H]-FdUMP-binding in some experiments (cf Table 2 and Table 1). The reason for this is not known, but cytosols used to derive data in Table 2 were prepared from large tumors (> 2 g), whereas those in Table 1 were from smaller tumors (~ 0.5 g). This may indicate that CH$_2$FH$_4$ concentrations decrease with increasing tumor mass.

After treatment of tumor-bearing mice with either FH$_4$ or FH$_2$, subsequent covalent binding of [6-^{3}H]-FdUMP to TS was decreased *in vitro*, even after the addition of CH$_2$FH$_4$. Since the principal folate component of mammalian blood and liver has been identified as 5-CH$_3$FH$_4$ [12, 19], it was possible that FH$_4$ and FH$_2$ were converted to this folate derivative *in vivo*, which is a non-competitive inhibitor of TS with respect to CH$_2$FH$_4$ [10]. This may explain the decreased formation of covalent ternary complex *in vitro*. The ability of 5-CH$_3$FH$_4$ to increase covalent binding of [6-^{3}H]-FdUMP *in vitro* by conversion to FH$_4$ was thus examined further by the addition of either homocysteine, B$_{12}$ or both to tumor cytosols. However, the addition of these two cofactors required in the conversion of 5-CH$_3$FH$_4$ by 5-CH$_3$FH$_4$:L-homocysteine methyltransferase (EC 2.1.1.13) did not increase the formation of the covalent complex. As the conversion of 5-CH$_3$FH$_4$ *in vitro* was apparently low, the fate of [5-^{14}CH$_3$]-FH$_4$ was subsequently examined *in vivo*. In liver, 83% of the radiolabel had been incorporated into protein 1 hr after treatment. This rate of conversion was similar to that reported in L1210 and L5178Y cells [25]. In tumor, however, only 17% of the [5-^{14}CH$_3$]FH$_4$ present was metabolized at 1 hr. Free [5-^{14}CH$_3$]-FH$_4$ was decreased in tumors by 4 hr, paralleling the decrease in total dpm/g of tissue. These data demonstrated that [5-^{14}CH$_3$]-FH$_4$ was not metabolized or retained to a great extent by tumors. In addition, no polyglutamate forms of [5-^{14}CH$_3$]-FH$_4$ were detected in tumor, although polyglutamylfolates have been demonstrated in neoplastic cells [11], human fibroblasts [26], intestine [27], cells of the blood [28, 29] and mammalian liver [12, 30–32]. In cultured human fibroblasts, intracellular polyglutamates were detected within 4 hr, but did not reach a maximum for about 10 days during incubation with [^{3}H]-FA [26]. Logarithmically growing cells also accumulated intracellular folate more rapidly and in higher concentrations than did confluent cells. In solid tumors, therefore, the proliferative state may influence the rate and extent of polyglutamate formation, which may be low under conditions *in situ*. In addition, the availability of L-glutamine, which has been demonstrated as necessary for the rapid formation of polyglutamates of methotrexate in cultured cells, may also influence the synthesis of these folate forms [33].

It remains to be demonstrated whether complex formation *in vitro* and that occurring in the tumor *in situ* are similar. Data presented in Table 2, for example, assume that enzymes involved in folate metabolism are functional under assay conditions. Data in Table 3 and in the HPLC analysis would suggest a rather low rate of 5-CH$_3$FH$_4$ conversion *in vitro* and *in vivo*. However, it would be preferable to measure the covalent complex formed *in situ*, after injection of folic acid derivatives. Such complexes have been isolated from cells growing in culture [34], although such techniques may prove more difficult in the intact animal due to rapid catabolism of [^{3}H]-FUra and low TS levels in tumors.

In human colon xenografts, sensitivity of tumors to FUra has not correlated with either its incorporation into RNA [4], levels of free FdUMP achieved [4], TS activity [5], the degree of inhibition of the incorporation of [6-^{3}H]-dUrd into DNA [35] or the actual concentrations of endogenous CH$_2$FH$_4$. Responsiveness to 5-fluorinated pyrimidines, however, does appear to correlate with the degree of inhibition of TS by FdUMP *in vitro* in the presence of endogenous cofactor [5]. The effect of the latter may be influenced by the accumulation of endogenous dUMP, which has approximated concentrations in the range of 2 mM in neoplastic cells after the inhibition of TS [8, 36]. This nucleotide is a potent inhibitor of the binding of FdUMP [16, 36]; thus, a low capacity to accumulate dUMP has correlated with FUra sensitivity in cultured cells [37]. The combined effects of dUMP and low cofactor concentration may be synergistic in their prevention of TS inhibition by FdUMP [16]. In colon xenografts, accumulation of dUMP in FUra-insensitive lines in addition to the formation of an unstable covalent complex may

prevent reassociation of the complex during the period when the levels of FdUMP are high (1 hr; [5]).

The formation of non-covalent ternary complexes with FdUMP or dUMP [21, 38] and TS have been reported with naturally occurring folate derivatives; their possible role in the inhibition of TS has also been suggested [16, 38]. Although covalent complex formation in tumor cytosols has not been increased by injection of folate derivatives into tumor-bearing mice, the role of non-covalent complex formation and the effects on the biosynthesis of dTMP appear to be interesting areas to probe in the elucidation of factors that may increase the responsiveness of tumors to FUra *in vivo*.

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