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DETERMINATION OF THYMIDYLATE SYNTHASE ACTIVITY IN COLON TUMOR TISSUES AFTER TREATMENT WITH 5-FLUOROURACIL*

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Abstract—The formation and isolation of [6-^{3}H]FdUMP-thymidylate synthase-5,10-methylenetetrahydrofolate covalent complex have been examined in tumor cytosols incubated with albumin–dextran coated charcoal used to remove endogenous nucleotide. Charcoal suspension (10% charcoal, 0.5% albumin, 0.05% dextran) adsorbed >98% of dUMP added to cytosols, but it reduced by 42–87% covalent complex isolated from subsequent incubation with [6-^{3}H]FdUMP and cofactor using cytosols from different tumors. Initial treatment of ternary complex with charcoal suspension did not cause a decrease in stability of covalent complex during subsequent incubation (37°), but complex separated from free ligand by 10% charcoal suspension was not stable to further treatment with 4% charcoal suspension. Treatment of tumor cytosols with 10% charcoal suspension, to remove nucleotide, did not decrease the rate at which enzyme catalyzed the release of ^3H_2O from [5-^{3}H]dUMP, or release active enzyme from the ternary complex. Based on these observations, a sensitive procedure for determining thymidylate synthase activity has been developed in which unbound nucleotides (dUMP, FdUMP) are removed prior to assay of enzyme activity. The procedure is suitable for assay of small samples of tissue or of tissues with a low (or inhibited) level of thymidylate synthase activity.

Thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase) catalyzes the reductive methylation of dUMP and, in mammalian cells, provides a unique pathway for dTMP biosynthesis *de novo*. This enzyme is of considerable importance to proliferating cells and is a target for several classes of anticancer agents. 5-Fluorouracil (FUra‡) is metabolized to FdUMP which forms a covalent ternary complex with thymidylate synthase in the presence of [6*R*]-CH_2-H_4PteGlu, thus inhibiting dTMP biosynthesis. Several studies have indicated that in tumors the level of thymidylate synthase prior to, or residual free thymidylate synthase subsequent to, administration of 5-fluoropyrimidines may be predictive of tumor response [1, 2]. Assays in which free thymidylate synthase has been determined, using [6-^{3}H]FdUMP as a specific ligand [3, 4], have been reported. However, accumulation of dUMP and unbound FdUMP may interfere with accurate determination of available ligand binding sites. Further, it is necessary to correct for exchange between bound FdUMP and free [^{3}H]FdUMP [1]. Fernandes and Cranford [5] recently described a modified ligand binding assay in which endogenous nucleotides were removed by treating cytosols with 10% charcoal suspension, thus removing potential interfering nucleotides. However, such treatment of cytosols derived from solid tumors causes a significant decrease in apparent binding of [^{3}H]FdUMP, without decreasing the metabolism of [5-^{3}H]dUMP. Failure to isolate radiolabeled complex appears to be due to instability during the second isolation with charcoal, albumin and dextran, and not a consequence of dissociation of ternary complex during incubation subsequent to the initial charcoal–albumin–dextran treatment used to remove unbound nucleotide. Based on these observations, a modified procedure using the assay of Roberts [6] for determining thymidylate synthase activity in treated or untreated tissue samples has been developed which should be of value for such measurement in tissues with low levels of thymidylate synthase.

MATERIALS AND METHODS

[6-^{3}H]FdUMP (sp. act. 20 Ci/mmole) and [5-^{3}H]dUMP (sp. act. 22 Ci/mmole) were purchased from Moravek Biochemicals (Brea, CA). Activated neutralized charcoal and dextran (average M_r 500 kD) were obtained through the Sigma Chemical Co. (St. Louis, MO). [6*RS*]-CH_2-H_4PteGlu was synthesized as described by Zakrzewski and Sansone [7] and stored at −80° under argon. Human tumors were grown as subcutaneous xenografts in immune-deprived mice [8]; these were $HxGC_3$, $HxELC_2$, and $HxVRC_5$ colon adenocarcinomas, and HxRh12/VCR, a rhabdomyosarcoma. Tumors were excised rapidly and disrupted using a Polytron homogenizer (Brinkmann Instruments, three times 15 sec) in ice-cold buffer [20 mM Tris–HCl, pH 7.5, containing

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‡ Abbreviations: FUra, 5-fluorouracil; [6*RS*]-CH_2-H_4PteGlu, racemic mixture of the natural [6*R*] and unnatural [6*S*] diastereoisomers of 5,10-methylenetetrahydropteroylmonoglutamate; and 10% charcoal suspension, 10% activated charcoal + 0.5% bovine serum albumin + 0.05% dextran.

70 mM β-mercaptoethanol, 100 mM NaF, 5 mM AMP (or 15 mM CMP), and 1.5 mM NaN_3]. Homogenates were centrifuged at 105,000 *g* for 60 min (4°), and supernatant fractions were used for assay.

Affinity purification of thymidylate synthase. Briefly, enzyme from $HxVRC_5$ xenografts was purified by fractionation of 100,000 *g* supernatant fractions using solid ammonium sulfate. The 30–70% precipitate was dialyzed, and thymidylate synthase was purified using Affigel Blue chromatography followed by elution from an affinity column of 10-formyl-5,8-dideazafolate-Sepharose (a gift from Dr. J. R. Bertino, Yale University School of Medicine) as described by Rode *et al.* [9]. The enzyme was subsequently dialyzed extensively, to remove dUMP which was a potential contaminant, and concentrated using an Amicon Concentrator with a YM10 membrane. This procedure gave >4000-fold purification. Thymidylate synthase was stabilized by addition of bovine serum albumin (1%) and stored at 4°.

Thymidylate synthase activity. The ^3H displacement assay of Roberts [6] was used, as described previously [10]. Standard conditions used 10 μM [5-^3H]dUMP (sp. act. 1.46 Ci/mmole) and 100 μM CH_2-H_4PteGlu. Reaction mixtures were incubated at 37° (2 min) prior to addition of radiolabeled substrate.

Dissociation of [^3H]FdUMP ternary complex. Ternary complex was formed in cytosols from $HxVRC_5$ xenografts by incubating at 37° (60 min) with 72 nM [^3H]FdUMP and 100 μM CH_2-H_4PteGlu. Complex was separated from unbound ligand by mixture with an equal volume of 10% charcoal suspension (ice-cold), centrifugation, and filtration through a glass fibre filter as described. In isolated complex >90% of radiolabel was precipitated by ice-cold 0.75 M perchloric acid (PCA). To determine the rate at which ternary complex dissociated, complex was incubated at 37°, and the increase in acid-soluble radioactivity was determined over time.

All charcoal suspensions contained 0.5% bovine serum albumin and 0.05% dextran.

RESULTS

The relationship between the volume and concentration of charcoal suspension required to adsorb nucleotide from tumor cytosols is shown in Fig. 1. In the presence of 15 mM CMP and 100 μM [5-^3H]dUMP, it was necessary to mix an equal volume of 10% charcoal suspension to remove >98% of added radiolabel. Thus, for subsequent assays cytosols were mixed on ice with an equal volume of ice-cold 10% charcoal suspension and were centrifuged (105,000 *g*, 20 min, 4°) to sediment charcoal. This supernatant fraction, or cytosol diluted with an equal volume of 0.5% albumin and 0.05% dextran, was incubated (37°, 30 min) with [6-^3H]FdUMP (72 nM) and [6*RS*]-CH_2-H_4PteGlu (100 μM), and covalent complex was isolated using a 5% charcoal suspension (Table 1). In cytosols derived from three different human tumors (two colorectal adenocarcinomas and one rhabdomyosarcoma), treatment with 10% charcoal suspension reduced by 42–87% the apparent formation of covalent complex. Whether charcoal treatment altered the formation of complex or its stability to subsequent isolation using 4% charcoal suspension was examined (Table 2). Covalent complex was formed with cytosols or purified enzyme and isolated from free [6-^3H]FdUMP using 10% charcoal suspension, or filtration through G-25 Sephadex. Complex was held on ice, and aliquots (150 μl) were mixed with 1.2 ml of ice-cold 4% charcoal suspension as before. After centrifuging (12,000 *g*, 4°, 10 min) and filtering through a fiber glass filter (to remove slight contamination with charcoal), radioactivity in filtrates was determined. Under these conditions, covalent complex formed with purified enzyme was unstable to a second treatment with 4% charcoal suspension, only 3% of the anticipated radiolabel remaining in the filtrate. Complex formed in $HxVRC_5$ cytosols was more stable to treatment with charcoal suspension (73%), whereas radiolabeled ternary complex isolated by gel filtration, which was >95% acid precipitable, was stable to charcoal treatment. These data may indicate that the higher protein concentration in $HxVRC_5$ cytosols (≈20 mg/ml) compared to purified enzyme (made 1% with BSA), acts to stabilize the ternary complex to albumin- and dextran-coated charcoal treatment. That treatment of cytosols with charcoal alters the stability of ternary complex to subsequent separation from free ligand, and not necessarily the formation of complex, is suggested by the observation that [5-^3H]dUMP metabolism is not reduced in treated cytosols (Fig. 2). The increase in reaction rate in $HxELC_2$ cytosols treated with 10% charcoal suspension was probably due to removal of endogenous dUMP, which is high in this tumor line (≈16 μM).

These results suggested that radiolabeled complex formed in cytosols pretreated with 10% charcoal suspension was unstable during the second charcoal isolation procedure. Alternatively, complex may have dissociated rapidly after initial charcoal treatment, thus liberating active enzyme. This would lead to an overestimate of residual enzyme activity in

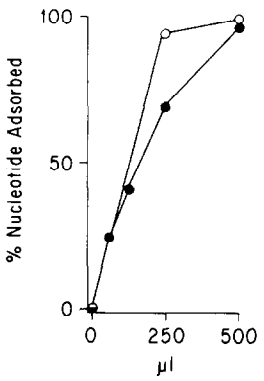

Fig. 1. Relationship between volume and concentration of charcoal suspension required to adsorb nucleotide from tumor cytosol. Aliquots (500 μl) of tumor cytosol (containing 100 μM [5-^3H]dUMP and 15 mM CMP) were mixed on ice with different volumes of 5% (●) or 10% (○) charcoal suspension. Volumes were adjusted to 1 ml with buffer, the charcoal was precipitated (12,000 *g*, 10 min, 4°), and the unbound radiolabel was determined. Each point represents the mean of duplicate determinations.

Table 1. Isolation of ternary complex formed in tumor cytosols

Tumor line	Complex isolated (dpm/mg protein) No charcoal	10% Charcoal suspension	% Isolated
$HxELC_2$	1.1×10^3	1.6×10^2	15
$HxGC_3$	3.8×10^3	2.2×10^3	58
HxRh12/VCR	2.2×10^4	2.6×10^3	13

Cytosols were mixed with an equal volume of 0.5% BSA + 0.05% dextran, or 10% charcoal + 0.5% BSA + 0.05% dextran. After removal of charcoal by centrifugation, complex was formed for 30 min (37°), using 72 nM [6-^{3}H]FdUMP and 100 μM [6*RS*]-CH_2-H_4PteGlu as described, and isolated using 5% charcoal suspension.

Table 2. Stability of isolated complex to treatment with albumin–dextran treated charcoal

Primary isolation procedure	Complex added to 4% charcoal suspension (dpm)	Complex recovered (dpm)	Recovery (%)
10% Charcoal suspension	43,345 ± 1,154	31,725 ± 1,882	73.2
G-25 Sephadex	2,264 ± 143	2,726 ± 161	120.4
	8,866 ± 257	8,103 ± 272	91.4
4% Charcoal suspension*	28,504 ± 1,308	875 ± 16	3.1

Ternary complex was formed with [6-^{3}H]FdUMP (125 nM), 100 μM [6*RS*]-CH_2-H_4PteGlu and $HxVRC_5$ tumor cytosol or affinity purified thymidylate synthase (120 min, 37°). Complex was isolated using charcoal or by removal of unbound ligand by chromatography through G-25 Sephadex (14.5 × 2.5 cm) equilibrated in homogenizing buffer. Aliquots (300 μl) of complex were maintained on ice and mixed with 1.2 ml of ice-cold 4% charcoal suspension. Complex was processed as described.

* Affinity purified thymidylate synthase from $HxVRC_5$ tumors.

extracts derived from FUra-treated samples. Stability of radiolabeled complex was examined subsequent to removal of free [^{3}H]FdUMP by treatment with 10% charcoal suspension (Fig. 3). After separation from charcoal, complex was incubated at 37° with or without addition of 100 μM CH_2-H_4PteGlu, and radiolabel appearing in the soluble fraction, after precipitation with PCA, was determined. The half-time for dissociation of complex (no added CH_2-H_4PteGlu) was 52 min. In samples where 100 μM CH_2-H_4PteGlu was added, >89% of radiolabel was precipitated by acid at 60 min. These data indicate that initial treatment of cytosol with 10% charcoal suspension did not cause an increased rate at which ternary complex dissociated.

To examine further whether charcoal treatment caused reactivation of inhibited enzyme, complex was formed using 1 μM FdUMP, 100 μM CH_2-H_4PteGlu (60 min, 37°), and isolated using 10% charcoal suspension. Alternatively, cytosol was incubated as above, without FdUMP, and mixed with an equal volume of 0.5% BSA, 0.05% dextran solution. Thymidylate synthase activity was determined in each sample (Fig. 4). The data show that >90% of enzyme activity was inhibited under these conditions.

Based upon the foregoing data a procedure has been developed for assaying thymidylate synthase activity in tumor samples. Tumors were homogenized in 2 vol. of ice-cold buffer, and cytosols were prepared as described. An equal volume of ice-cold 10% charcoal suspension was added, the mixture recentrifuged (105,000 *g*, 20 min, 4°), and this supernatant retained on ice. To determine thymidylate synthase activity, the reaction mixture contained 110 μl supernatant fraction, 10 μM [5-^{3}H]dUMP (sp. act. 1.9 Ci/mmole), 100 μM [6*RS*]-CH_2-H_4PteGlu in a total volume of 150 μl. Reactions were initiated by adding preincubated cytosol (37°, 2 min) to prewarmed reactants and were terminated at 10 min by removing 40-μl aliquots and mixing with 10 μl of 0.75 M perchloric acid on ice. To this 1.2 ml of ice-cold 4% charcoal suspension was added, and the mixture was incubated on ice for a further 15 min. Charcoal was removed by centrifugation (12,000 *g*, 4°, 5 min) and filtration through a glass fiber filter (Gelman GF/A) packed into a disposable 1-ml syringe. Radioactivity was determined in 800 μl of filtrate. Using this procedure, thymidylate synthase activity in xenografts of a human colon adenocarcinoma was determined before and at various times after administration of FUra (100 mg/kg; Fig. 5). These data show that there is a rapid, but relatively transient depression of thymidylate synthase activity in these xenografts, which are intrinsically resistant to FUra.

DISCUSSION

Measurement of thymidylate synthase in specimens of human colon adenocarcinoma presents considerable problems due to the low level of this enzyme in such tissues. Such technical problems are exacerbated in samples exposed to FUra, where enzyme may be inhibited. Several methods used to determine total, free and bound thymidylate synthase [3–5] based upon [^{3}H]FdUMP ligand binding

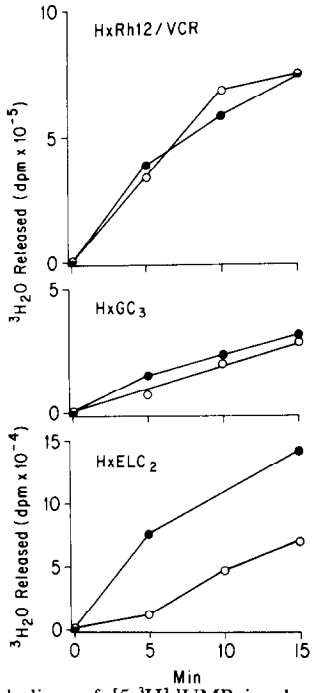

Fig. 2. Metabolism of [5-^{3}H]dUMP in charcoal-treated cytosols. Tumor cytosols were mixed with an equal volume of 10% charcoal suspension (●) or 0.5% albumin + 0.05% dextran solution (○), and incubated on ice for 1 min. Charcoal was precipitated, and thymidylate synthase activity was determined in the supernatant fractions. HxRh12/VCR is a human rhabdomyosarcoma, and $HxELC_2$ and $HxGC_3$ are human colon adenocarcinoma xenografts. Results are the mean of duplicate determinations for each point.

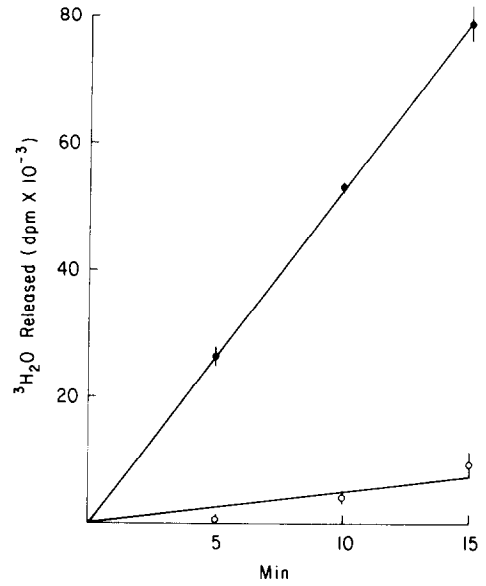

Fig. 4. Activity of thymidylate synthase after isolation of ternary complex using 10% charcoal suspension. Ternary complex was formed with non-labeled FdUMP and isolated from unbound ligand as described. Key: thymidylate synthase activity in isolated complex (○), and activity in an equivalent volume of uninhibited cytosol (●). Each value is the mean ± 1 SD (N = 3).

assays have been proposed. The assay derived by Moran *et al.* [4] has been used to quantitate thymidylate synthase in murine colon adenocarcinomas before and after treatment of tumor-bearing mice with FUra [1]. For such quantitation, however, results had to be corrected for [^{3}H]FdUMP isotope dilution by free FdUMP present in cytosols; hence, concomitant determination of FdUMP was necessary. Fernandes and Cranford [5] proposed a modified procedure in which dissociation of bound enzyme was allowed to proceed in the presence of dUMP (to

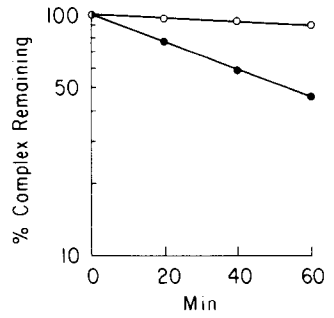

Fig. 3. Stability of [^{3}H]FdUMP ternary complex subsequent to treatment with 10% charcoal suspension. Complex was isolated, as described in Materials and Methods, and incubated at 37° in the absence (●) or after the addition of 100 μM CH_2-H_4PteGlu (○). Aliquots were removed and precipitated with perchloric acid. At zero time >90% of radiolabel was precipitated. Release of [^{3}H]FdUMP from ternary complex was monitored by determining radiolabel in the acid-soluble fraction. Results are the mean from three determinations (SD < 5% mean).

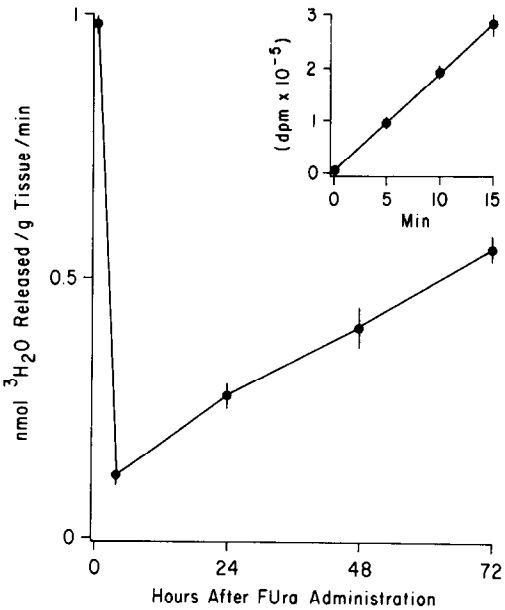

Fig. 5. Thymidylate synthase activity determined in $HxGC_3$ xenografts before and 4, 24, 48 and 72 hr after administration of FUra (100 mg/kg) to tumor-bearing mice. Four tumors were pooled to produce cytosols at each time point. Each point represents the mean ± SD for three determinations. Inset: linearity of the reaction in cytosols from control tumors.

prevent reassociation of FdUMP), and free nucleotide and reduced folate were removed by addition of an ice-cold 10% charcoal suspension. Free enzyme was quantitated in a similar manner, but without the period of incubation to allow dissociation. This modification, because unbound nucleotide is removed, negates the requirement for measurement of FdUMP in cytosols. Further, interference of [^{3}H]FdUMP binding by dUMP, which may accumulate in human tissues subsequent to FUra administration [10], will be reduced. When we applied this procedure to measure thymidylate synthase levels in extracts derived from xenografts of human colon adenocarcinoma, it was evident that preadsorption of nucleotide using 10% charcoal suspension led to greatly reduced [^{3}H]FdUMP binding when cytosols were incubated subsequently with radiolabeled ligand (Table 1). This appeared to be a consequence of the initial treatment of cytosol by charcoal, as complex separated by gel filtration from unbound [^{3}H]FdUMP was stable to subsequent isolation with 4% charcoal suspension. Of interest was that this initial adsorption step did not reduce the activity of thymidylate synthase as determined by [5-^{3}H]dUMP assay [6]. Preadsorption of cytosol with 10% charcoal suspension, removed free nucleotide without causing dissociation of the ternary complex (>90% [^{3}H]FdUMP could be precipitated by acid). However, to be of value it was necessary to demonstrate that preadsorption did not cause the ternary complex to dissociate rapidly during the subsequent incubation with [5-^{3}H]dUMP. Subsequent to adsorption with charcoal suspension, the rate of net dissociation of ternary complex was relatively slow ($T^{1/2}$ = 52 min, 37°) in the absence of added CH_2-H_4PteGlu. This is slower than the rate of dissociation subsequent to filtration of complex on G25-Sephadex ($T^{1/2}$ = 33 min) [11] and may indicate that preadsorption with 10% charcoal suspension does not remove entirely unbound CH_2-H_4PteGlu used to form complex. In the presence of 100 μM CH_2-H_4PteGlu, charcoal isolated complex was quite stable (Fig. 4). Further, free enzyme was not released during the subsequent incubation to determine residual thymidylate synthase activity.

We have combined the charcoal suspension preadsorption procedure of Fernandes and Cranford [5] to remove unbound FdUMP or accumulated dUMP without disrupting the covalent ternary complex (note that without albumin and dextran complex was dissociated, data not shown) with the sensitive isotopic assay of Roberts [6], for determination of the activity of thymidylate synthase. Using this procedure it was shown that, *in vivo*, there was a rapid, but relatively transient depression of thymidylate synthase activity in $HxGC_3$ colon adenocarcinoma xenografts, which are intrinsically resistant to FUra [12]. The assay procedure described is both sensitive and reproducible and has application for tissues in which [^{3}H]FdUMP binding is low. In the presence of saturating CH_2-H_4PteGlu concentration (100 μM), dissociation of complex is negligible during the period for assay of enzyme activity (10–15 min) and, because the end-point is determined by substrate metabolism, i.e. turnover (rather than binding of FdUMP to the enzyme), may prove more sensitive than current [^{3}H]FdUMP binding assays. For the colon adenocarcinomas examined, less than 25% of [^{3}H]dUMP was metabolized during the assay period. However, in order to prevent depletion of substrate, dUMP concentration could be increased to 20 μM (maintaining the same specific activity). Alternatively, the assay period could be reduced for tumors having high activity of thymidylate synthase. The procedure determines also the parameter that may more readily determine response of colon adenocarcinomas, that is, the residual catalytic activity of the rate-limiting enzyme in the synthesis *de novo* of thymidine 5′-phosphate.

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