In Vivo Kinetics of Thymidylate Synthetase Inhibition in 5-Fluorouracil-sensitive and -resistant Murine Colon Adenocarcinomas


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ABSTRACT

The predictive utility of several biochemical parameters of 5-fluorouracil (5-FUra) action was evaluated in four murine colonic adenocarcinomas: 5-FUra-sensitive Tumor 38 and 5-FUra-resistant Tumors 07/A, 51, and 06/A. Thymidylate synthetase (TS) was determined by a tritiated 5-fluoro-2′-deoxyuridylate (FdUMP)-binding assay. Bolus 5-FUra (80 mg/kg, i.p.) administration caused in all tumors a rapid decrease in free TS levels. Only Tumor 38, however, showed inhibition of TS to undetectable (<0.05 pmol/g) levels, which lasted up to 6 hr after treatment; correction for dissociation of endogenous TS: FdUMP:folate ternary complex during the TS assay was required. Total TS (free enzyme plus ternary complex) was determined with experimental conditions that achieved quantitative recovery of free TS from ternary complex. By 48 hr after 5-FUra, Tumor 38 showed a decrease in total TS proportional to the estimated log kill/dose of 5-FUra; in contrast, the resistant tumors showed no such decrease from pretreatment levels. Assay of FdUMP showed that the free nucleotide was formed rapidly in all tumors in excess over available TS-binding sites. However, tumor sensitivity did not correlate with peak or residual FdUMP levels or with deoxyuridylate levels, which were low and remained so in all tumors. Tumor sensitivity to 5-FUra also could not be explained by the small differences among the tumors in total perchloric acid-soluble metabolites of 5-FUra or drug incorporation into RNA. We conclude from these data that levels of free TS in the tumor after 5-FUra treatment are predictive of chemotherapeutic response in these murine models of human colonic adenocarcinoma.

INTRODUCTION

The antitumor drug 5-FUra3 is widely used as a palliative treatment for disseminated internal cancers and as curative therapy for several kinds of epithelial neoplasms (1, 9). The principal mechanism of cytotoxicity of 5-FUra has long been thought to result from the inhibition of TS, which provides the therapeutic importance of a second site of action of 5-FUra, namely, its effects on RNA metabolism. The most important of these effects is probably drug incorporation into RNA and consequent inhibition of RNA maturation (17). Several investigators have observed decreased drug incorporation into RNA in 5-FUra-resistant cells, together with decreased total acid-soluble 5-FUra metabolites (predominantly ribonucleotides) (3,

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3 The abbreviations used are: 5-FUra, 5-fluorouracil; TS, thymidylate synthetase; FdUMP, 5-fluoro-2′-deoxyuridylate; dUMP, 2′-deoxyuridylate; CH₂FH₄, 5-10-methylene tetrahydrofolate; TSₘₜₐₜ, total thymidylate synthetase; TS, free non-5-fluoro-2′-deoxyuridylate-bound thymidylate synthetase; TSₜₜ, ternary complex 5-fluoro-2′-deoxyuridylate-bound thymidylate synthetase; FH₄, L- (+)-tetrahydrofolate; TS, experimentally determined [³H]5-fluoro-2′-deoxyuridylate-binding sites.

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Materials. [6-3H]FdUMP and 5-[6-3H]FUra were purchased from Moravek Biochemicals, Brea, Calif. [14C]Formaldehyde was purchased from New England Nuclear, Boston, Mass. Purified Lactobacillus casei TS and FH, were prepared as reported (33). Purified TS from human CCRF-CEM lymphoblasts (29) was a gift of Dr. Arnold Lockshin. FdUMP was a gift of Dr. Peter V. Danenberg. DEAE-cellulose was obtained from Eastman Organic Chemicals, Rochester, N. Y. 5-FUra was from Roche, Inc., Nutley, N. J. All other reagents were obtained from Sigma Chemical Co., St. Louis, Mo. Buffer A consisted of 0.6 M NaH2PO4, pH 8.0, containing 100 mM 2-mercaptoethanol, 100 mM NaF, and 15 mM cytidylate. Buffer B consisted of 50 mM potassium phosphate buffer, pH 7.4, with 20 mM 2-mercaptoethanol, 100 mM NaF, 15 mM cytidylate, and 2% bovine serum albumin. Cofactor solution consisted of Buffer B which also contained 2 mM FH4, 16 mM sodium ascorbate, and 9 mM formaldehyde. All buffers were sterilized by passage through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.).

Tumor Models. The 4 colon adenocarcinomas of this study originated in BALB/c mice (Tumors 51, 06/A, and 07/A) or C57Bl/6 mice (Tumor 38) following s.c. 1.2-mg hydroxyurea hydrochloride (15). Histologically, they all showed intermediate grades of differentiation, although Tumor 51 was mucin producing. The tumors were passaged in the host of origin by axillary transplantation and were studied at transplant generations 18 to 23 (Tumor 38), 17 to 23 (Tumors 07/A), 75 to 80 (Tumor 51), and 21 to 27 (Tumor 06/A).

For chemotherapy sensitivity experiments, Tumor 38 was transplanted into C57Bl/6 x DBA/2 (hereafter called BD2F,) mice and the other 3 tumors were transplanted into BALB/c x DBA/2 (hereafter called CD2F,) mice. Tumor size was calculated using length and width of the tumors. The tumors were serially distributed into the host by use of a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.).

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separate ternary complex from charcoal-bound FdUMP and cofactor. A 50-μl aliquot of the 4000 × g supernatant was added to 50 μl of Buffer A, and the TS, \text{app} activity was then determined as above by addition of [3H]FdUMP and cofactor solution, as a function of time at 30° before addition of charcoal.

**Incorporation of 5-FUra into RNA.** In separate experiments, mice were given 5-[6-3H]Fu (7 mCi/mmol) at 80 mg/kg. The perchloric acid-, lipid-extracted pellets were digested by KOH and assayed for RNA and DNA as above. Although lipid-solvent-extractable radioactivity was negligible, omission of the organic solvent step markedly reduced RNA recovery. Radioactivity associated with DNA was regularly less than 1% of that present in tissue sonicates. The low level of radioactivity used in this study permitted the routine performance of TS, FdUMP, and dUMP assays, without correction for increased background.

**RESULTS**

**Tumor Response to 5-FUra Treatment.** Colon Tumor 38 showed moderate sensitivity to 5-FUra given i.p. at maximally tolerated dosage to mice bearing relatively advanced (about 200-mg) s.c. tumors. The indices of therapeutic response, the percentage increase in life span and the tumor growth delay shown in Table 1, are only somewhat lower than values resulting from 5-FUra treatment of early-stage (about 60-mg) disease and are also similar to results obtained with 5-fluorodeoxyuridine (8). The more relevant parameter may be the tumor growth delay time, which is analogous to the period of clinical remission that describes response duration in patients. In the other 3 tumors, 5-FUra did not show significant evidence of chemotherapy activity, although colon Tumor 07/A had been modestly responsive at earlier transplant generations (data not shown).

Sensitivity to 5-FUra did not correlate with tumor growth rate or metastatic potential. Tumor volume-doubling times were 3.4, 2.7, 4.2, and 3.2 days for colon Tumors 38, 07/A, 51, and 06/A, respectively, and were similar to growth rates observed in the mice used for passage. In the latter animals, this corresponded to an average radial growth of 0.35 ± 0.13 (S.E.) mm/day for the 4 tumor types. In separate studies of untreated mice with far-advanced disease, the incidence of gross pulmonary metastasis was found to decrease in the order Tumor 51 (100%), Tumor 06/A (>75%), Tumor 38 (<50%), and Tumor 07/A (<20%).

**Labeling of TS by [3H]FdUMP.** Chart 1 shows the results of measuring the rate of exchange of [3H]FdUMP into preformed unlabeled ternary complex in the TS assay, using the 105,000 × g cytosolic enzyme obtained from untreated CCRF-CEM cells (33), colon Tumors 38 and 06/A, or a human breast adenocarcinoma. Control values (i.e., total [3H]FdUMP-binding sites) in these experiments were obtained from the results of tubes carried in parallel but without the addition of nonlabeled FdUMP. At high CH$_3$FH$_4$ concentration, the rate of appearance of radioactivity in ternary complex, $k_r$, is governed by the rate of ternary complex dissociation, which is slow compared to the rate of ternary complex formation (11, 29, 43). Linear regression by least-squares analysis of the combined data up to 90 min ($N = 23$; $r = 0.9633$) showed a $k_r$ value of $6.37 \times 10^{-3}$ min$^{-1}$. Thus, regardless of the tumor source, about 13% of TS$_b$ dissociated and rapidly reformed ternary complex containing [3H]FdUMP during the 20-min TS assay incubation period and appeared as TS$_i$. TS$_i$ was therefore calculated from the following relationship:

$$TS_i = TS_{tot} - TS = (TS_{app} - 0.13 TS_{tot})/0.87$$

where TS$_{app}$ is the experimentally determined concentration of [3H]FdUMP-binding sites present at the end of 20 min in the standard TS assay. The $k_r$ value for CCRF-CEM TS at pH 7.4 was $4.14 \times 10^{-3}$ min$^{-1}$, or 65% of the rate at pH 8.0.

The determination of TS$_{tot}$ is therefore highly useful for increased accuracy in the assay of TS$_i$. This is particularly true if the enzyme is predominantly in the form of the ternary complex. Assay of TS$_{tot}$ depends on the full recovery of native FdUMP-binding sites from the ternary complex by complete

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**Table 1**

<table>
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<tr>
<th>Tumor</th>
<th>%ILS$^a$</th>
<th>T - C$^d$</th>
<th>$\log_{10}$ kill/dose</th>
<th>Pretreatment</th>
<th>Nadir</th>
<th>TS$_{tot}$ at 48 hr</th>
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<td>06/A</td>
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<td>7.5</td>
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<td>38</td>
<td>58</td>
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<td>0.25</td>
<td>32.1</td>
<td>$\pm 0.05^b$</td>
<td>16.9</td>
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</table>

$^a$ s.c. tumors (20- to 30-mg transplantation size) were allowed to grow to 100- to 300-mg size range prior to 5-FUra treatment.

$^b$ 5-FUra given at the maximally tolerated dosage of 50 mg/kg/day i.p. a total of 4 times at 4-day intervals beginning Day 11 (Tumor 06/A, Day 15 (Tumor 51), and Day 13 (Tumors 07/A and 38) after transplantation.

$^c$ TS responses based on single-dose 5-FUra given 80 mg/kg i.p. on Day 21 after transplantation.

$^d$ Percentage increase in life span, based on median survival after transplantation compared to control mice (N = 10 in each group); control mice lived 34 days (Tumor 38), 57 days (Tumor 07/A), 99 days (Tumor 51), and 34 days (tumor 06/A).

$^e$ Tumor growth delay, treated minus control (T - C): the time lag, in days, for treated tumors to reach a predetermined size (1250 mg) compared to controls; the median time to reach 1250 mg in control mice was 25 days (Tumor 38), 22 days (Tumor 07/A), 39 days (Tumor 51), and 21 days (Tumor 06/A).

$^f$ Log$_{10}$ kill/dose = [(T - C) × log 2 /Td × number of doses], where Td = the tumor volume-doubling time, in days, at the 100- to 400-mg size range.

$^g$ Represents the lower limit of TS$_i$ detection.
dissociation of the complex during the 3-hr preincubation period. For the determination of TS\(\text{bound}\), no added cofactor is present in the preincubation mixtures, in which cytosolic folates present are lowered 90% by dilution. Low preincubation reduced folate concentrations were expected to decrease the rate of ternary complex formation so that equilibrium at 3 hr favored the TS\(\text{i}\) state. To demonstrate the absence of appreciable ternary complex formation at low preincubation FH4 concentrations, 0.38 pmol of TS from untreated CCRF-CEM cells was labeled with 6 pmol of \(\text{[3H]FdUMP}\) in a final volume of 250 \(\mu\)l using standard preincubation conditions, except that added FH4 was varied from 0 to 1 mm final concentration and 1.0 mm formaldehyde and 1.6 mm ascorbate were present. At the end of 3 hr, protein-bound radioactivity (determined by charcoal absorption) obeyed the relationship, TS\(\text{b}/\text{TSi} = [\text{FH4}] / 51.3 \mu M\), which was linear \((r = 0.9995; N = 10)\) down to 0.5 \(\mu M\) FH4. Thus, for example, at a FH4 concentration of 0.5 \(\mu M\) in the preincubation mixture using colon tumor cytosols, only 1.0% of the available enzyme sites would have been bound by cytosolic FdUMP (assuming \([\text{FdUMP}] \geq [\text{TS}]\)).

Several lines of experimental evidence indicate that full recovery of enzyme in the TS\(\text{i}\) state occurs during the 3-hr preincubation. (a) Treatment of tumor cytosols with 10% neutral charcoal before enzyme assay permitted separation of ternary complex(es) and TS\(\text{i}\) from endogenous folates and nucleotides; assay of charcoal-treated 4000 \(\times g\) supernatant fractions for TS\(\text{i}\) and TS\(\text{tot}\) at all time points after 5-FUra treatment revealed no significant differences from results obtained without treatment of these cytosols with charcoal. (b) Examination of neutral charcoal-treated cytosols of Tumors 38 and 51, 1 hr after 5-FUra administration, showed that the rates of increase in \(\text{[3H]FdUMP}\)-binding sites during the preincubation period, after the period of rapid labeling of free enzyme, were \(6.86 \times 10^{-3}\) \(\text{min}^{-1}\) and \(6.22 \times 10^{-3}\) \(\text{min}^{-1}\), respectively. These rates are indistinguishable from \(k_\text{b}\) values obtained using ternary complex formed with FH4 prior to \(\text{[3H]FdUMP}\) addition. Thus, under our TS assay conditions, the contribution of cytosolic folylpolyglutamates to \(k_\text{b}\) appears to be negligible. (c) Extension of the preincubation period to 4 hr caused less than a 3% increase in TS\(\text{b}\) in these tumors. (d) There was no significant difference between TS\(\text{i}\) and TS\(\text{tot}\) analyzed in cytosols from untreated tumors, and the differences between pretreatment TS\(\text{i}\) values and TS\(\text{b}\) at short times after 5-FUra were quite small (see below). Therefore, we conclude that TS\(\text{tot}\) determinations at short times after 5-FUra exposure yield excellent approximations of pretreatment TS\(\text{i}\) values.

**In Vivo Kinetics of TS Inhibition.** The results of TS\(\text{i}\) and TS\(\text{tot}\) determination for the 4 colon tumors are shown in Charts 2 and 3 and are summarized in Table 1. Pretreatment values of TS\(\text{i}\) showed wide variation among the tumor lines but did not correlate with the response to 5-FUra or with the biological behavior of the tumors. However, Tumor 06/A, possibly the most 5-FUra resistant (Table 1), possessed 4 to 6 times higher pretreatment TS\(\text{i}\) values than the other tumors.

Within the first hr after 5-FUra administration, all tumors showed a profound decrease in TS\(\text{i}\) (Chart 2). Most striking was the TS\(\text{i}\) reduction found for 5-FUra-sensitive colon Tumor 38, in which TS\(\text{i}\) was undetectable during the period 0.5 to 6 hr after 5-FUra administration. Colon tumor 38 also showed the lowest TS\(\text{i}\) of the 4 tumors at 12 and 24 hr following treatment. The time course of the increase in TS\(\text{i}\), subsequent to initial TS\(\text{i}\) inhibition was similar for the tumors with similar pretreatment TS\(\text{i}\) levels, namely, Tumors 38, 07/A, and 51. The increase in TS\(\text{i}\) at 48 hr was greatest for Tumor 06/A, which had the highest pretreatment TS\(\text{i}\). From the pretreatment growth rates and TS\(\text{i}\) values of these tumors, it can be calculated that up to one-half of the regeneration of TS\(\text{i}\) seen in Chart 2 is attributable to new tumor growth.

The 24- and 48-hr TS\(\text{tot}\) levels in 5-FUra-sensitive colon Tumor 38 were about one-half of its pretreatment level (Chart 3). In marked contrast, tumor TS\(\text{tot}\) either increased or showed no significant change from pretreatment values through 48 hr following 5-FUra administration to mice bearing the resistant colon tumors 06/A, 51, and 07/A. The decrease in TS\(\text{tot}\) of Tumor 38 was due to a decrease in TS\(\text{b}\), since the TS\(\text{i}\) steadily increased after initial inhibition. The TS\(\text{i}\) decrease was not offset by an equivalent increase in TS\(\text{b}\) and therefore could not be solely attributable to intracellular ternary complex dissociation. A likely cause of this decrease in TS\(\text{i}\) in Tumor 38 was cell death, since the estimated log\(\text{d}_{10}\) kill/dose of 0.25 (Table 1) predicted a 44% decrease in viable cell number and hence, presumably, a corresponding decrease in TS. The early, tran-
sient increases in TSmax observed in Tumors 07/A and 38 were suggestive of a cell synchrony effect (38). These data shown in Charts 2 and 3 were not appreciably changed when normalized by tissue DNA content.

**Nucleotide Assays.** No correlation was found between the sensitivity to 5-FUra (Table 1) and either peak or persistent levels of FdUMP (Chart 4). Intracellular FdUMP formation in the 4 murine colon tumors was rapid. The highest levels of the free nucleotide were found at the earliest time point evaluated, 0.5 hr. All tumors showed a sharp decrease in free FdUMP concentration at 1 hr, synchronous with the inhibition of TS and formation of ternary complex. In Tumors 38 and 51, however, ternary complex formation could not account for most of the free FdUMP losses. The ratio of peak FdUMP to pretreatment TS generally correlated with the nadir of TSt inhibition. However, this ratio was highest for Tumor 51, which showed incomplete TS inhibition. Free FdUMP persisted at low levels (<60 pmol/g) throughout the period of study; in Tumors 06/A and 07/A, free FdUMP after 6 hr was consistently lower than available TS-binding sites.

Studies with [3H]FdUMP-labeled ternary complex showed that, although ternary complex was extracted into the acetic acid supernatant, 0.0% and <0.2% of the radioactivity chromatographed with the dUMP and FdUMP fractions on DEAE-cellulose, respectively. Hence, our method of estimation of free FdUMP excludes FdUMP bound to enzyme.

Pretreatment dUMP levels in the 4 murine tumors were similarly low (3 to 8 nmol/g) and did not increase following 5-FUra treatment (Chart 5).

**Nucleic Acid Content.** DNA contents of colon Tumors 38, 07/A, 51, and 06/A were respectively 19.9, 21.3, 9.8, and 12.5 mg/g, wet weight. RNA contents were 8.1, 7.5, 4.2, and 6.6 mg/g, respectively. These values are similar to those of normal rodent intestine (27).

**Incorporation of 5-FUra into RNA.** Colon Tumor 38 showed a slightly higher concentration of total perchorlic acid-soluble 5-[3H]FUra metabolites than the other tumors, at 3, 12, and 24 hr after drug administration (Table 2). However, this small difference was not apparent when the results were normalized on the basis of tumor RNA content. Peak acid-soluble metabolite levels were found at the earliest post-5-FUra time point studied, 3 hr, for all tumors except 06/A, which showed fairly constant metabolite levels through 48 hr. The perchloric acid-soluble metabolites accounted for approximately 90% of the total radioactivity found in the crude sonicates.

The specific activities of RNA isolated from Tumors 38, 07/A, and 51 were not significantly different. The highest levels of 5-FUra incorporation into RNA of these tumors were at the earlier time points and represented about 1 fluororibonucleotide base/600 ribonucleotides. Colon Tumor 06/A showed slower drug incorporation into RNA, which paralleled the slower rate of TS inhibition and slower acid-soluble metabolite formation in this tumor. 5-FUra incorporation into RNA was a higher proportion of total acid-soluble metabolites (25%) in Tumor 07/A than in Tumor 38 (14%), Tumor 51 (9%), or Tumor 06/A (12%). There was no obvious relationship between 5-FUra incorporation into RNA and FdUMP levels, although differences among the tumors in any case were not great.

**DISCUSSION**

5-FUra-sensitive colon Tumor 38 showed quantitative and qualitative differences from the 3 resistant tumors in the time course of inhibition of TS (Charts 2 and 3). TS was undetectable up to 6 hr after chemotherapeutic 5-FUra administration in Tumor 38; standard deviations showed no overlap with TS values of the 5-FUra-resistant tumors during this time period. The resistant tumors never had less than 1.9 pmol of TS per g, which apparently was sufficient to allow pretreatment rates of tumor growth. The rate of return of TS levels after initial inhibition was similar in the 4 tumors and was probably twice the rate attributable to renewed or continued tumor growth alone. Intracellular enzyme in Tumor 38 was entirely in the inactive TSmax state up to 6 hr after 5-FUra treatment. By 48 hr, TSmax in Tumor 38 declined 47% from its pretreatment level, in agreement with the estimated log10 kill/dose of 0.25, or 44% cell kill/dose (Table 1). These results are compatible with our hypothesis that reduction in TS to growth rate-limiting levels
ultimately resulted in death of cells containing high amounts of TS relative to TS levels. High amounts of TS per se would not be expected to correlate with cytotoxicity, as shown by the results in Tumor 06/A.

Although the ternary complex among TS, FdUMP, and folate cofactor is covalently linked, the reaction that results in complex formation is reversible (11). This implies that dissociation of the complex in the cell would allow a recovery of enzyme activity in the presence of excess dUMP as has been discussed previously (2, 33, 36, 37). Moreover, this reversibility has major practical importance for the measurement of TS by any technique that employs either Vmax determination in the presence of excess dUMP or labeling of active sites with [3H]-FdUMP. Thus, spectrophotometric (47) or tritium release (3, 12, 42) assays of enzyme activity in the presence of substantial levels of TS will overestimate TS, unless the results are extrapolated to true initial rates. Likewise, titration of TS with [3H]FdUMP (19, 33, 43) must be corrected for the rate of TS dissociation that occurs during the period of exposure to [3H]-FdUMP, since exchange of [3H]FdUMP with FdUMP in TS occurs at a modest rate (Chart 1). Hence, the technique discussed in this report for the determinations of TStot and TS on a single sample allows an accurate estimate to be made of how much enzyme was, in fact, free for de novo synthesis of thymidylate in the tissue. Our previous estimates of TS in 5-FUra-inhibited CCRF-CEM cells had suggested that approximately 20% of the TS content of these cells was present as TS in spite of the presence of excess FdUMP; in view of the data of Chart 1, this clearly was an overestimate. The potential for error in TS determinations is illustrated by our observation that the storage of colon tumor cytosols at 4°C for several days in the absence of added CH2FH4 can lead to nearly complete dissociation of TS (data not shown).

The increased efficiency of TS inhibition in Tumor 38 was not a result of particularly high peak formation or persistence of FdUMP (Chart 4), whose levels were highest at the earliest time studied (0.5 hr) and reflected the rapidly changing balance among events of 5-FUra transport and activation and loss of free FdUMP by catabolism and binding to TS. The rapid FdUMP losses in all tumors within an hr of treatment were similar to the kinetics described recently in P388 cells in vivo (3) and human carcinoma cells in vitro (12). At most time points, the highest free FdUMP contents were found in Tumor 51, although at 48 hr Tumor 38 showed the highest FdUMP, 34 pmol/g. The late rise of free FdUMP in Tumor 38 may have been caused by ternary complex destruction during cell death in vivo, resulting in release of extracellular FdUMP. In the resistant tumors, the coexistence of free FdUMP and TS at early time points suggests that intracellular conditions did not allow complete ternary complex formation or stabilization.

Tissue dUMP contents of these tumors corresponded to cytosol dUMP concentrations in the 5 to 10 μM range and, therefore, were probably too low to have slowed rates of ternary complex formation (29, 36). In addition, differences among the dUMP levels of these tumors were too small to explain the greater TS inhibition in the 5-FUra-sensitive Tumor 38. In contrast to our previous results in human CCRF-CEM cells continuously exposed to 5-FUra (33), these murine colon adenocarcinomas did not show appreciable dUMP accumulation after 5-FUra treatment.

In comparison with normal colon tissue, colon Tumor 38 may have somewhat higher fluorouracil phosphorobisyltransferase activity (6), which has provided an explanation (44) for the apparently enhanced therapeutic index when 5-FUra is preceded by allopurinol administration in this tumor. However, our study of 5-FUra metabolism to total acid-soluble metabolites (which presumably are predominantly fluororibonucleotides) and incorporation into RNA revealed no differences significant enough to explain the 5-FUra sensitivity of colon Tumor 38, although the slow rates of these events in Tumor 06/A may have been an additional factor in the resistance of this tumor.

Adequate 5-FUra transport, activation to nucleotide(s), and incorporation into RNA may still be insufficient events per se to cause therapeutically useful sensitivity to 5-FUra at maximally tolerated bolus i.p. doses. Lowering of TS to levels approaching zero may be necessary for growth inhibition by 5-FUra. Marked TS inhibition requires tight FdUMP binding to enzyme. At FdUMP concentrations equivalent to or exceeding TS, a critical variable that determines ternary complex stability is the concentration of reduced folates. Although nearly all TS studies have used CH2FH4, a monoglutamyl form, this folate probably is not the active cofactor for this enzyme intracellularly (32). Intracellular folates exist as polyglutamates (5, 34, 46), which may differ from CH2FH4 in their kinetics of interaction with TS (10, 13, 23, 28). Washtien and Santi (48) have shown that considerably slower ternary complex dissociation occurs in intact cells in culture than in high CH2FH4-containing cell-free cytosols. It thus seems likely that differences in the concentration of the reduced folates in these colon tumors could be a determining factor in causing inhibition of TS in tumoricidal levels. This hypothesis is supported by the recent suggestion of Houghton et al. (19) that cytosol of 5-FUra-resistant human colorectal xenografts required the addition of exogenous CH2FH4 in order for increased FdUMP binding to occur.

In conclusion, our study has provided evidence that tumor sensitivity to 5-FUra correlates with immediate and complete inhibition of TS. Dissociation of ternary complex is sufficiently facile that active enzyme may be overestimated (3, 12, 33). We have found that correction for such dissociation is essential for the accurate determination of active TS in vivo after exposure to 5-FUra. Other indices of 5-FUra action that we studied (incorporation into RNA, dUMP levels, FdUMP levels, total

Table 2

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* Time of sacrifice (hr) after [6-3H]FUra.
b Average ± S.E. of [6-3H]FUra (7 mCi/mmole) given i.p. at 80 mg/kg to 3 tumor-bearing mice/time point.

Kinetics of TS Inhibition

Table 2

<table>
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<th>Total acid-soluble 5-FUra metabolites (nmol/g)</th>
<th>Incorporation of 5-FUra into RNA (nmol/mg RNA)</th>
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<td>3.9 ± 1.6</td>
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<tr>
<td>129 ± 12</td>
<td>4.0 ± 1.6</td>
</tr>
<tr>
<td>56 ± 9</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>80 ± 12</td>
<td>3.9 ± 1.6</td>
</tr>
</tbody>
</table>
acid-soluble metabolites) did not correlate with 5-FUra sensitivity in these tumors.

ACKNOWLEDGMENTS

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