Biochemical Determinants of 5-Fluorouracil Response In Vivo

THE ROLE OF DEOXYURIDYLATE POOL EXPANSION

CHARLES E. MYERS, ROBERT C. YOUNG, and BRUCE A. CHABNER

From the Laboratory of Chemical Pharmacology and the Medicine Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20014

ABSTRACT 5-Fluorodeoxyuridine monophosphate (FdUMP), the active metabolite of 5-fluorouracil (5-FU), is a tight-binding inhibitor of thymidylate synthetase, the enzyme which converts dUMP to TMP. Newly developed assays for FdUMP and dUMP were utilized to assess the competitive roles played by these nucleotides in determining the inhibition of TMP synthesis in mice bearing the P1534 ascites tumor. After 5-FU administration, levels of FdUMP reached a dose-dependent peak within 6 h in the ascites tumor and in bone marrow, and declined thereafter in a biphasic manner with an initial $t_{\frac{1}{2}}$ of 6 h and a final $t_{\frac{1}{2}}$ of 7-9 days. In duodenal mucosa, FdUMP levels were 1.8–2-fold higher than in the other tissues, but elimination was much more rapid. Simultaneous with the fall in FdUMP a progressive accumulation of the competitive substrate dUMP was observed in each tissue after 5-FU; and peak dUMP levels coincided with recovery of thymidylate synthesis, as determined by the incorporation of [^{3}H]deoxyuridine into DNA.

In vitro experiments with partially purified thymidylate synthetase revealed an initial competitive interaction of dUMP and FdUMP, which, at high concentrations of dUMP was capable of markedly slowing the rate of irreversible inactivation of enzyme by FdUMP. These studies were found to be quantitatively consistent with a two-phase model of enzyme inactivation involving an initial competition between dUMP and FdUMP, with subsequent irreversible inactivation of enzyme by covalent linkage to the inhibitor. Recovery of thymidylate synthesis after 5-FU appears to result from both a fall in intracellular levels of inhibitor and a progressive accumulation of the competitive substrate dUMP.

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INTRODUCTION

The inhibition of thymidylate synthetase by 5-fluorodeoxyuridylate (FdUMP)[1] is believed to underlie the antineoplastic and myelotoxic effects of 5-fluorouracil (5-FU) (1). A central question in understanding the action of this agent, and the designing of combination therapy with 5-FU is the elucidation of the events which determine the duration of inhibition of thymidylate synthetase and which lead to recovery of this pathway in normal and neoplastic tissue.

In initial studies of its mechanism of action, FdUMP appeared to act as a classic competitive inhibitor of thymidylate synthetase; this hypothesis was supported by the finding that deoxyuridine reversed the inhibition of DNA synthesis in tissue culture, presumably by augmentation of the intracellular pools of the natural substrate, deoxyuridine monophosphate (dUMP) (2, 3). However, recent studies utilizing purified thymidylate synthetase from mammalian (4) and bacterial (5) sources have demonstrated that FdUMP binds covalently to the active site of the target enzyme. The resulting FdUMP-enzyme complex does not dissociate significantly during protein denaturation with trichloroacetic acid, 6 M urea, 6 M guanidine or chromatography on sodium dodecyl sulfate gel. In addition, digestion of the complex with pronase yields a small polypeptide with FdUMP still firmly attached (6). These results indicate that the binding of FdUMP to thymidylate synthetase in vitro is essentially irreversible, an observation which is not easily reconciled with classical kinetics of competitive enzyme inhibition (7, 8).

The present studies were undertaken to clarify the role of sequential changes in substrate (dUMP) and inhibitor (FdUMP) concentrations which are associ-

[1] *Abbreviations used in this paper:* CH_2FH_4, methylene-5,10-tetrahydrofolate; FdUMP, 5-fluorodeoxyuridylate; 5-FU, 5-fluorouracil; TdR, thymidine; UdR, deoxyuridine.

ated with the suppression and recovery of thymidylate synthetase activity after 5-FU. These studies, based on newly described assays for FdUMP and dUMP (9), strongly support the concept of an initial competitive interaction of these nucleotides in determining activity of the thymidylate synthetase pathway.

Further in vitro experiments have been carried out in an attempt to resolve the conflict between the known stability of the FdUMP-enzyme complex and the apparent competitive reversal observed in vivo. These studies have revealed a marked effect of dUMP concentration upon the rate at which FdUMP binds to thymidylate synthetase, presumably through occupying the enzyme active site. This evidence has led us to formulate a model for recovery from inhibition of thymidylate synthetase based on protection of newly synthesized thymidylate synthetase by the increased pools of dUMP.

METHODS

5-FU, dUMP, and tetrahydrofolic acid were obtained from Sigma Chemical Co., St. Louis, Mo. [6-^{3}H]Deoxyuridine (10 Ci/mmol), [*methyl*-^{3}H]thymidine (6.7 Ci/mmol), and Aquasol were obtained from New England Nuclear, Boston, Mass. [5-^{3}H]dUMP (17.6 Ci/mol) was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. CDF_1 mice, 18–24 g, were obtained from the National Institutes of Health Breeding Colonies, caged in a controlled-temperature environment, and allowed water and Purina Lab Chow ad libitum. Experiments were carried out during the same time period each day. The source and characteristics of the P1534 tumor line used in this study has been previously described (9). For the dUMP and FdUMP assays, thymidylate synthetase, prepared from dichloromethotrexate-resistent *Lactobacillus casei* by the method of Crusberg et al. (10), was obtained from New England Enzyme Center, Tufts University Medical School, Boston, Mass. The enzyme preparation formed 5.5 mmol dTMP/h per mg protein at pH 7.0 and 30°C.

Preparation of mammalian thymidylate synthetase. 150 CDF_1 mice were injected i.p. with 1×10^6 P1534 ascites tumor cells. On the 4th day of tumor growth, the mice were killed and the ascites tumor cells removed by lavage of the peritoneal cavity with iced phosphate-buffered saline. The resulting cell suspension was centrifuged at 9,000 *g* for 10 min. After resuspension in twice its volume of 0.05 M Tris-HCl buffer, pH 7.1, 0.01 M mercaptoethanol, and 0.001 M EDTA (buffer A), the cells were subjected to two cycles of freeze-thawing, following by Dounce homogenization. The resulting cell-free suspension was centrifuged at 90,000 *g* for 60 min. The supernate was purified according to the method of Fridland et al. (11) through fraction II. The resulting enzyme preparation contained 24 mg of protein per ml and formed 0.512 nmol dTMP/h per mg protein at 37°C.

Thymidylate synthetase assay. Mammalian thymidylate synthetase was assayed by following the release of tritium from [5-^{3}H]dUMP (12). The radiochemical purity of the dUMP was checked by chromatography on precoated cellulose chromatographic plates (Analtech, Inc., Wilmington, Del.) with isopropanol : concentrated NH_4OH : 0.1 M boric acid (6 : 1 : 3). Approximately 50 μCi of labeled dUMP was placed in each of several 12 × 75-mm glass tubes, lyophilized, and stored at −70°C until needed. This approach minimized the spontaneous exchange of tritium with solvent during storage. The enzyme was assayed at 25°C in an 80-μl volume containing 20 μl of enzyme and the same concentrations of buffer A, and methylene-5-10-tetrahydrofolate (CH_2FH_4) as described in the legend to Fig. 5B. The reaction was initiated with the addition of 40 μl of 2.5×10^{-3} M dUMP (2.6 mCi/mmol). After 30 min, the reaction was terminated with 10 μl of 40% TCA followed by the addition of 10 μl of unlabeled dUMP (95 mg/ml) and 200 μl of a charcoal slurry (Darco, 100 mg/ml, Atlas Chemical Industries, Inc., Wilmington, Del.). The sample was centrifuged at 4,000 *g* for 3 min and 150 μl of the supernate removed for liquid scintillation spectrophotometry in Aquasol. Because there is a significant exchange of tritium between [5-^{3}H]-dUMP and water which is increased by Tris buffer and mercaptoethanol (13), a control reaction lacking enzyme was run with every determination. In this paper, 1 U of enzyme activity was defined as the amount required to synthesize 1 μmol of thymidylate per min at 30°C in Tris-HCl buffer (pH 7.1).

Effects of 5-FU on incorporation of [^{3}H]thymidine (TdR) and [^{3}H]deoxyuridine (UdR) into DNA in vivo. CDF_1 mice, weighing 18–25 g, were inoculated i.p. with 1×10^6 P1534 ascites tumor cells, and on the 4th day thereafter were treated with 5-FU in doses of 15, 50, or 100 mg/kg i.p. The dosage range chosen ranged from a dose causing no prolongation in survival (15 mg/kg) to one prolonging survival from a control median value of 7 days to 9.5 days.[2] At specified time intervals after drug administration, six mice each received 100 μ Ci i.p. of either [^{3}H]UdR or [^{3}H]TdR. 1 h after the administration of labeled nucleoside, at a time when TdR and UdR incorporation into DNA were maximal (14, 15), the mice were killed and ascitic tumor cells were obtained by lavage of the peritoneum with iced phosphate-buffered saline (0.85% NaCl, pH 7.4). A 5-cm section of duodenum was removed, slit longitudinally, the mucosa scraped from the muscularis with a glass slide and dispersed in iced buffered saline. Bone marrow cells were harvested from tibias by inserting a needle into one end of the medullary cavity and expelling the marrow with iced phosphate-buffered saline. Acid-soluble nucleotides including dUMP were extracted with 1 M acetic acid and saved for later analysis. DNA was extracted from the insoluble material by the method of Schneider (16). The DNA content of a 0.5-ml portion of the extract was then measured by the Burton method (17). A second portion of 0.5 ml was dissolved in Aquasol and ^{3}H content was determined by liquid scintillation spectrophotometry; the results were corrected for counting efficiency with an internal standard and expressed as disintegrations per minute per microgram DNA. These results were compared with uptake of label into DNA in untreated tumor-bearing animals at time zero, and expressed as percent of the control incorporation. The control values for deoxyuridine incorporation into DNA were 16,091±813 dpm/μg DNA for P1534 ascites tumor cells and 59±6.3 dpm/μg DNA for duodenal mucosa. The control values for thymidine incorporation into DNA were 11,944±750 dpm/μg DNA for P1534 ascites tumor cells, 198±35 dpm/μg DNA for the bone marrow, and 301±32 dpm/μg DNA for the duodenal mucosa.

The values of deoxyuridine incorporation for each time point represented the average of four separate experiments, and each experimental determination was performed on

[2] Myers, C. Unpublished observations.

pooled tissues from three animals. The pooling of tissue was necessary to obtain sufficient material for analysis. The individual values from separate experiments were in close agreement: the standard error of the mean was less than 10% through the first 2 days and less than 15% for the remainder of the study. Because of the low level of deoxyuridine incorporation observed in the duodenal mucosa (control values of 59 dpm/μg DNA) incorporation values of less than 10% were not considered quantitatively significant.

Assay of dUMP and FdUMP pools after 5-FU. Aliquots of the acetic acid extracts of host tissue and tumor obtained in the nucleotide incorporation studies were then lyophilized to remove acetic acid and were reconstituted in 0.5 ml of distilled water. The characteristics of this acetic acid extraction technique have been previously described (9). The dUMP content of an aliquot of the sample were assayed by making dUMP the limiting substrate in the thymidylate synthetase reaction and monitoring the formation of product by following the increase in absorbance at 340 nm (9). An occasional sample formed a fine precipitate when added to the assay cuvette. To circumvent this problem, the sample and the volume of buffer to be used in the assay were combined, heated to 90°C for 5 min, and centrifuged at 40,000 *g* for 10 min to remove precipitate.

An additional aliquot of the sample was assayed for FdUMP as described in a previous report from this laboratory (9). This technique measures free FdUMP, not that already bound to the thymidylate synthetase in the tissue sample. The lower limit of sensitivity of this assay is 1 pmol in the assay cuvette. High dUMP concentrations in the tissue sample can interfere with the assay; we have previously reported 9×10^{-6} M as the upper limit of dUMP allowable in the assay cuvette. However, because the binding of FdUMP to thymidylate synthetase in the presence of dUMP is time dependent, this upper limit may be extended to 5×10^{-5} M by preincubation of enzyme, CH_2-FH_4, and sample for 20 min at 25°C before assay of residual enzyme activity. The SE's of the dUMP determinations were less than 15% for the first 2 days and less than 20% for the remainder of the study. For the FdUMP determinations, the SE was less than 20% for the first 36 h and less than 25% for the remainder of the study.

Correction of [^{3}H]UdR incorporation for dUMP pool size. 5-FU induces marked changes in the intracellular levels of dUMP. Therefore, the measured incorporation [^{3}H]UdR into DNA is a function both of the rate of dUMP conversion to dTMP and changes in the endogenous dUMP pools size. To correct for these pool size changes, incorporation of [^{3}H]UdR into DNA was multiplied by the fractional change in the nucleotide pool size: fractional UdR incorporation at time x =

$$\frac{\text{dpm}/\mu\text{g DNA at time } x}{\text{dpm}/\mu\text{g DNA at time 0}} \times \frac{\text{dUMP}/\mu\text{g DNA at time 0}}{\text{dUMP}/\mu\text{g DNA at time } x}$$

The effect of this correction on the results of a typical experiment involving P1534 ascites tumor cells is illustrated in Fig. 1.

Analysis of the role of dUMP in recovery of thymidylate synthetase activity in vivo. The kinetics of binding of FdUMP to thymidylate synthetase was analyzed in the present experiments according to the model for tight-binding inhibition of acetyl cholinesterase by diisopropylfluorophosphate (18). This model assumes an initial stage of competition of inhibitor and substrate for the enzyme active site, with nondissociable attachment of inhibitor to enzyme at a

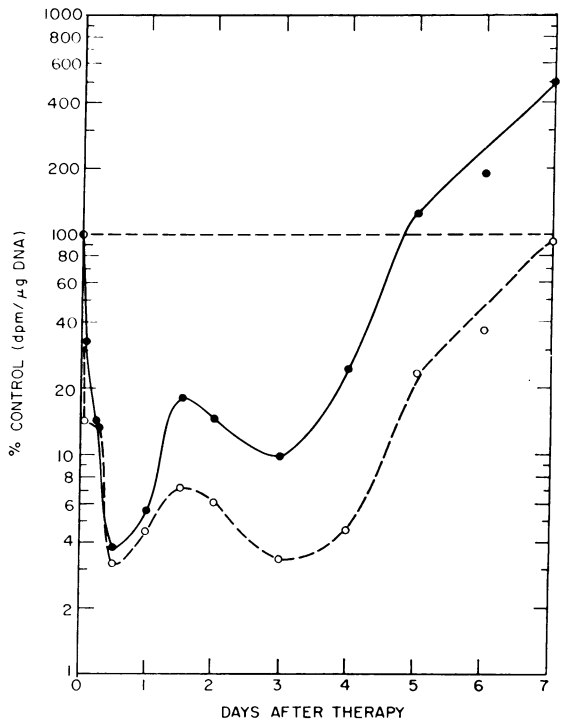

FIGURE 1 A comparison of uncorrected (○) and corrected (●) [^{3}H]UdR incorporation into DNA of P1534 ascites tumor cells after 100 mg/kg of 5-FU.

rate determined by the relative concentrations of competitors. In such a model, the kinetics of inhibition is expressed by the following equation:

$$\log \frac{1}{\alpha} = \frac{2.3K_a[I]t}{1 + \frac{[S]}{K_m}}, \qquad (1)$$

where K_a is the bimolecular rate constant; K_m is the Michaelis constant of S; α is the fraction of initial enzyme activity remaining; t is the time elapsed after inhibitor addition; and $[I]$ and $[S]$ are the inhibitor and substrate concentrations, respectively. Inspection of Eq. 1 reveals that for such tight binding inhibitors (*a*) the rate of inactivation should decrease rapidly as $[S]$ exceeds the K_m and (*b*) for any given combination of $[I]$ and $[S]$, the degree of inactivation should be a first-order function with respect to time.

It can be seen from Eq. 1 that as $[S]$ exceeds the K_m, the quantity $(1 + [S]/K_m)$ approaches $[S]/K_m$, and Eq. 1 simplifies to $\log 1/\alpha = 2.3K_aK_m[I]t/[S]$ *or* $\log \alpha = -2.3K_aK_m[I]t/[S]$. Thus, under conditions where $[S]$ exceeds the K_m, the velocity of enzyme inactivation is a first-order function of $[I]/[S]$. Such a relationship must be fulfilled in vivo if the rate of enzyme inactivation is the major determinant of the residual enzyme activity. In the present study $[I]$ and $[S]$ have been directly measured, and α may be approximated by the [^{3}H]UdR incorporation into DNA.

RESULTS

The correlation between alterations in dUMP and FdUMP concentrations and recovery of UdR incorporation after 5-FU. In order to verify the selective effect of 5-FU on thymidylate synthetase, preliminary studies were performed to compare the incorporation [^{3}H]UdR and [^{3}H]TdR into DNA of tumor cells, bone marrow, and duodenal mucosa. In all three tissues, a marked fall in [^{3}H]UdR incorporation was observed after 100 mg/kg of 5-FU i.p., with [^{3}H]TdR incorporation reaching peak values of 180%, 240%, and 240% of control levels within 6 h of drug administration in bone marrow, duodenal mucosa, and tumor, respectively. By 24 h deoxythymidine incorporation had returned to control levels in each of the three tissues studied. These findings are consistent with earlier reports indicating that 5-FU administration results in a specific block in thymidylate synthesis with preferential utilization of exogenous thymidine for DNA synthesis (19, 20). In further experiments, the incorporation of [^{3}H]UdR into DNA was utilized as an index of the degree of inhibition of the thymidylate synthetase pathway, and changes in activity of this pathway were related to the concentrations of intracellular substrate and inhibitor.

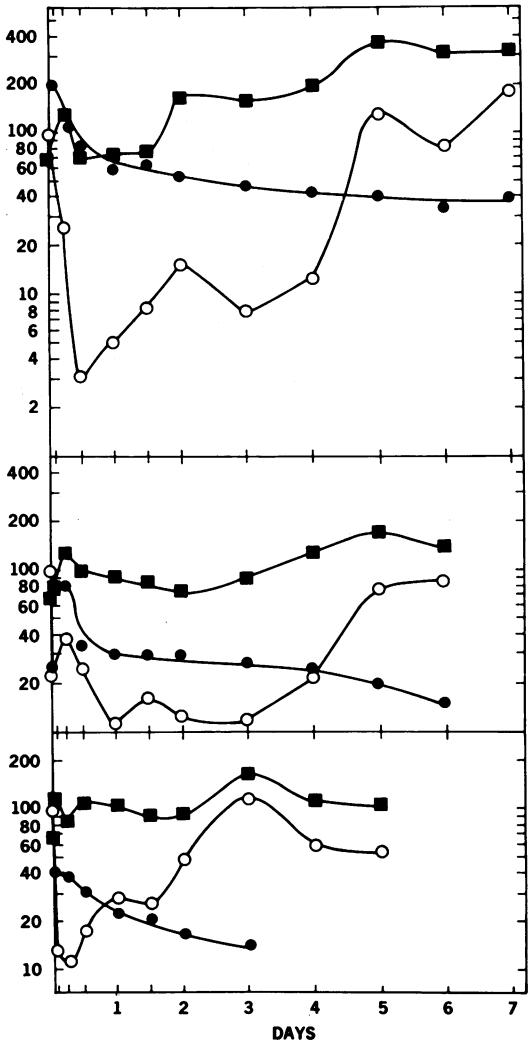

FIGURE 2 Correlation between FdUMP (●, femtomoles per microgram DNA) and dUMP (■, picomoles per microgram DNA) levels and the rate of UdR incorporation (○, percent control) in P1534 ascites tumor cells. CDF_1 mice bearing P1534 ascites tumors received 100 (top), 50 (middle), or 15 (bottom) mg/kg of 5-FU i.p.

Fig. 2 illustrates that after administration of doses of 15, 50, or 100 mg/kg, the incorporation of [^{3}H]UdR into P1534 ascites tumor fell to very low levels within 6 h, with recovery beginning at 36, 72, and 96 h, respectively. At all three dosage levels intracellular FdUMP in the ascites tumor reached a maximum within 6 h; and thereafter, the disappearance curve for free FdUMP was at least biphasic, with an initial $t_{\frac{1}{2}}$ of 6 h and an estimated final slow, $t_{\frac{1}{2}}$ of 7-9 days. The dUMP pool rapidly expanded during the first 6 h after 5-FU treatment, fell or plateaued for a short period of time thereafter, and then exhibited a sustained rise to between 2.5 and 6 times control levels depending on the dose of 5-FU. Peak tumor dUMP levels were attained coincident with recovery of [^{3}H]UdR incorporation into DNA.

The incorporation of [^{3}H]UdR into bone marrow DNA (Fig. 3) also decreased rapidly after 5-FU administration. Recovery occurred earlier than in tumor, beginning at 6, 12, and 48 h after doses of 15, 50, 100 mg/kg and was associated with a much more rapid and marked accumulation of intracellular dUMP than seen in the ascites tumor cells, reaching maximum values of 3.8–21-fold control levels at the time of recovery of [^{3}H]UdR incorporation into DNA. In contrast, the FdUMP levels were similar to those observed in the ascites tumor, reaching peak values within 6 h. Thereafter, the FdUMP disappearance curve was again biphasic with a rapid $t_{\frac{1}{2}}$ of 6-12 h and a slow $t_{\frac{1}{2}}$ of 2-5 days. Thus, in bone marrow, rapid recovery of [^{3}H]UdR incorporation coincided with the rapid increase in intracellular concentration of dUMP rather than an accelerated disappearance of free FdUMP.

As with the ascites tumor and bone marrow, incorporation of [^{3}H]UdR incorporation into DNA duodenal mucosa decreased rapidly after 5-FU administration, reaching a nadir within 6-12 h (Fig. 4). Recovery, which began within 24 h of drug administration at all three dosage levels, was followed by an overshoot phase of lesser magnitude than observed in the bone marrow. The synthesis of FdUMP was prolonged in this tissue,

continuing for up to 24 h after the 100-mg/kg dosage level. As a result, much higher peak levels of free FdUMP were attained in this tissue than in bone marrow or ascites tumor. Thereafter, elimination of FdUMP was rapid, and from 48 h on, FdUMP levels were characteristically lower in the duodenal mucosa than in either of the other tissues. The duodenal mucosal cell dUMP pool decreased briefly after 5-FU administration

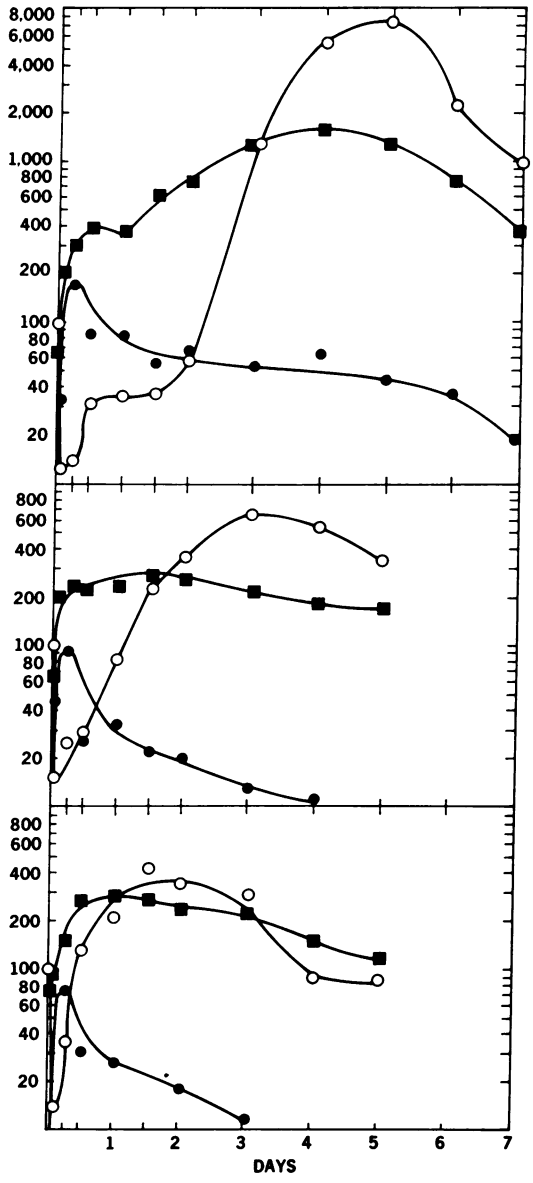

FIGURE 3 Correlation between FdUMP (●, femtomoles per microgram DNA) and dUMP (■, picomoles per microgram DNA) levels and the rate of UdR incorporation (○, percent control) into DNA of the bone marrow after 100 (top), 50 (middle), or 15 (bottom) mg/kg of 5-FU i.p.

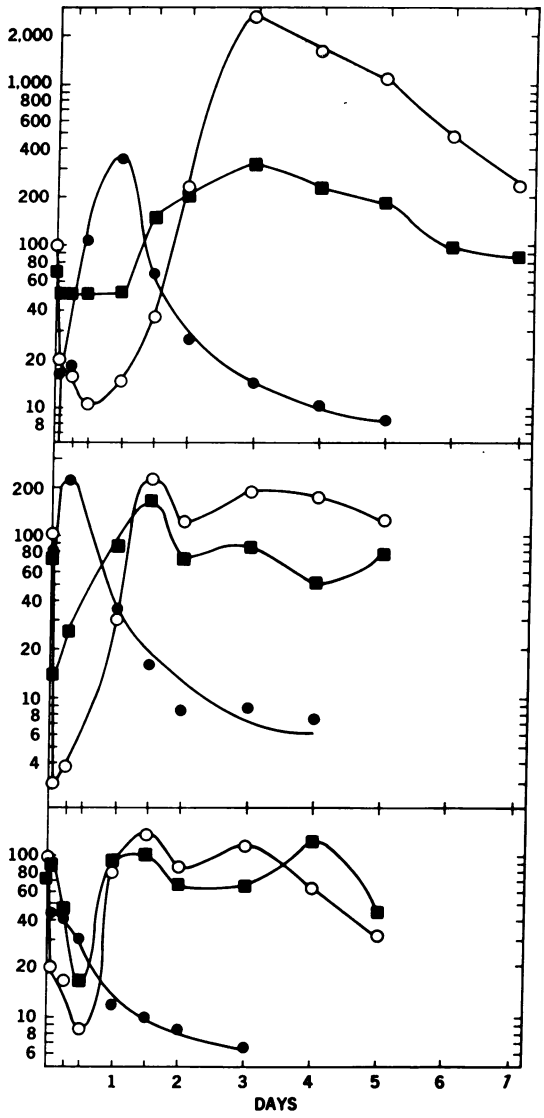

FIGURE 4 Correlation between FdUMP (●, femtomoles per microgram DNA) and dUMP (■, picomoles per microgram DNA) levels and the rate of UdR incorporation (○, percent control) into the DNA of duodenal mucosa after 100 (top), 50 (middle), or 15 (bottom) mg/kg of 5-FU i.p.

before rising above control levels and only at the highest dose, 100 mg/kg, was marked accumulation of dUMP observed. Nevertheless, the highest dUMP levels, 1.4–4.2 times control levels, again coincided with peak levels of [^{3}H]UdR incorporation.

Effect of substrate on enzyme-FdUMP complex formation. While these in vivo studies indicate an important role for dUMP accumulation in the recovery of DNA synthesis after 5-FU administration, they do not provide

direct observations on the competitive interaction of FdUMP and dUMP. This competitive interaction has been investigated further in vitro by applying a kinetic model developed to describe the progressive competitive tight-binding inhibition of cholinesterase by organophosphates (18). Details of this model have been presented in the Methods section.

Preliminary studies revealed that while inhibition of the enzyme by FdUMP in the absence of dUMP was virtually instantaneous; the rate of inactivation of the enzyme slowed progressively with increasing concentrations of dUMP, essentially ceasing when dUMP levels exceeded 10^{-3} M. This observation has allowed us to terminate the progressive inhibition of the enzyme by FdUMP through the addition of excess dUMP. Utilizing this approach, we have followed the time-course of the inhibition reaction in the presence of a wide range of dUMP concentrations (Fig. 5A). Comparison of these results with the kinetic equation for competitive tight-binding inhibitors (Eq. 1) reveals that the interaction of FdUMP and dUMP with thymidylate synthetase is consistent with this model in several respects. The rate of enzyme inactivation is dependent on dUMP concentrations and is first order with respect to time. Further, from the slope of each line in Fig. 5A and the K_m for dUMP as determined in Fig. 5B, it is possible

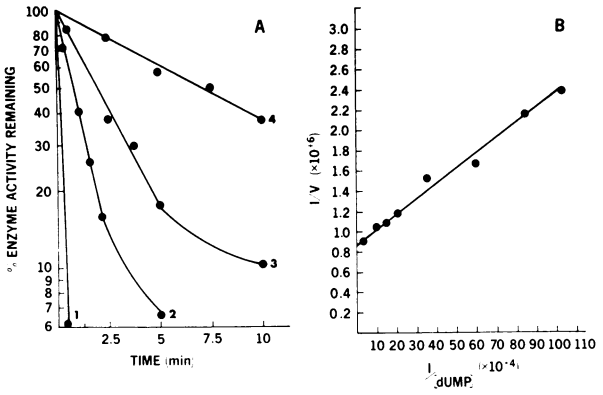

FIGURE 5 Influence of dUMP concentration upon the kinetics of enzyme inactivation of FdUMP. In Fig. 5A, 4 mU of enzyme (20 μl) was added to 45 μl of buffer A containing CH_2FH_4, 1.2×10^{-4} M; FdUMP, 1.2×10^{-7} M; bovine serum albumin, 1 g/100 ml; and dUMP, 0 (exp. 1), 1.34×10^{-6} M (exp. 2), 1.34×10^{-5} M (exp. 3), 1.34×10^{-4} M (exp. 4). After incubation at 25°C for specified time intervals, 40 μl of [5-^{3}H]dUMP (2.5×10^{-3} M, 2.6 mCi/mmol) was added to designated tubes and remaining enzyme activity assayed by tritium displacement assay. In Fig. 5B, 4 mU of enzyme (20 μl) was added to 80 μl buffer A containing CH_2FH_4, 7.6×10^{-4} M, and a variable concentration of [5-^{3}H]dUMP (50 μCi/mmol). Samples were incubated at 25°C for 10 min before termination with 40% TCA.

to calculate an association constant, K_a, for FdUMP from the above equation. The result obtained, $2.5 \pm 1.5 \times 10^7$ M^{-1} min^{-1}, is in close agreement with the value of 1×10^7 M^{-1} min^{-1} estimated by previous workers in the absence of dUMP utilizing rapid sampling techniques (5). Thus, the in vitro interaction of dUMP and FdUMP appears to be entirely consistent with the model for competitive tight-binding inhibition previously described for acetylcholinesterase.

The role of dUMP in recovery from effects of 5-FU in vivo. In order to evaluate whether the results obtained in the in vivo experiments were consistent with the competitive mechanism described in Eq. 1, the relationship between dUMP/FdUMP and [^{3}H]UdR incorporation was determined for the experiments in Figs. 2, 3, and 4, and the results are given in Fig. 6. In spite of marked differences in the actual time-course of changes in nucleotide concentration in the three tissues, the quantitative relationships between deoxyuridine incorporation and the FdUMP/dUMP ratio were all quite similar. However, the results approach the linear relationship predicted from Eq 1 (see Methods) only when the nucleotide ratio falls below 2×10^{-4}. This analysis supports the concept that in the tissues studied, recovery from 5-FU is determined both by the falling concentration of the inhibitor, FdUMP, and by changes in the level of the normal substrate, dUMP, but that the relationship is more complex than was suggested by Eq. 1.

DISCUSSION

The present report represents an analysis of the biochemical events which are associated with the recovery of [^{3}H]UdR incorporation into DNA after administration of 5-FU. This analysis has revealed that expansion of the endogenous dUMP pool is a characteristic response of the three tissues studied. The mechanisms whereby dUMP pools increase after 5-FU have not been defined by this study, but the increase is probably due to several factors. The first is likely to be decreased utilization of dUMP in the thymidylate synthetase reaction as the result of inhibition by FdUMP. A second may be derepression of deoxycytidylate deaminase activity due to decreased dTTP, with resulting increased conversion of dCMP to dUMP, a sequence of changes for which there is evidence after methotrexate administration (21, 22). Finally, decreased feedback inhibition both of *de novo* pyrimidine synthesis and ribonucleotide reductase activity as the result of decreased nucleoside triphosphates are other possible sources of increased dUMP synthesis (23). The considerable differences in the time-course of dUMP generation observed in the three tissues after 5-FU administration suggest, as was predicted by Werkheiser et al. (24), that the regulation

of deoxynucleotide pools may differ significantly from tissue to tissue.

The role of dUMP in determining the impact of 5-FU upon a tissue is supported by several lines of evidence: (*a*) in vitro, dUMP clearly effects the rate of binding of FdUMP to thymidylate synthetase; (*b*) in the recovery period following 5-FU administration, peak [^{3}H]UdR incorporation coincided temporally with peak dUMP levels; (*c*) the more rapid recovery of [^{3}H]UdR incorporation seen in bone marow as compared to the P1534 ascites tumor correlated with a more rapid accumulation of dUMP in the former, rather than in any differences in the rate of FdUMP elimination. In addition, previous investigators have shown that in tissue culture exogenous UdR reverses 5-FU's inhibition of DNA synthesis, presumably through augmentation of the endogenous dUMP pool (3).

The accumulation of dUMP observed in vivo may reverse inhibition of [^{3}H]UdR incorporation by two mechanisms. One possibility would be competitive displacement of FdUMP by dUMP, resulting in regeneration of previously inactivated enzyme. Such competitive displacement has been demonstrated in vitro by Reyes and Heildelberger (2). However, under most experimental conditions, dissociation of the FdUMP complex has proved negligible and the relative importance of this process remains to be established (4, 5). As illustrated in Fig. 5A, the rate at which FdUMP binds to thymidylate synthetase in vitro is determined by the concentrations of both FdUMP and dUMP. From this observation, it is possible to invoke a second mechanism (Fig. 7) to explain the effect of dUMP accumulation. Instead of regeneration of previously inactivated enzyme, this model postulates that the concentration of active enzyme available to sustain dTMP synthesis represents a balance between the processes of enzyme synthesis and enzyme inactivation; as FdUMP levels decline and the dUMP pool expands, the rate of enzyme inactivation and synthesis would shift in favor of recovery of enzyme activity toward normal. While the present study presents no direct evidence as to the relative contribution of either mechanism, the linear relationship observed in Fig. 6 between the FdUMP/dUMP ratio and the [^{3}H]UdR incorporation during the later stages of recovery supports the concept that dUMP may exert its major influence on the velocity of enzyme inactivation in vivo. A third possible process, increased synthesis of new enzyme, may play a role and cannot be excluded by these studies.

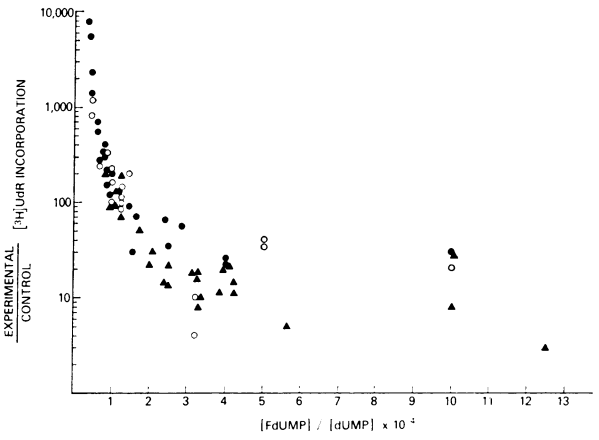

FIGURE 6 The correlation between the recovery of deoxyuridine incorporation and the ratio of nucleotide concentrations. The [^{3}H]UdR incorporation is expressed as (experimental/control) × 100. The solid circles represent the bone marrow; the open circles, the duodenal mucosa; and the solid triangles, the ascites tumor. Values below 10% for the duodenal mucosa were not included in this graph (see Methods).

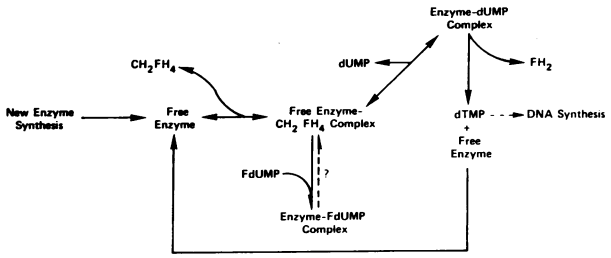

FIGURE 7 A model for the biochemical events determining the net concentration of active thymidylate synthetase after 5-FU.

Before incorporation into DNA, deoxyuridine must first undergo dilution in the endogenous dUMP pool. In the present study, the measured incorporation of deoxyuridine into DNA has been corrected for the marked alterations observed in the dUMP pool size. This procedure does not, however, account for additional dilution of the label by dTMP synthesized via the salvage pathway for thymidine. The resulting depression in [^{3}H]UdR incorporation would lead to an underestimation of the remaining thymidylate synthetase activity likely to be most prominant during periods of maximal suppression of thymidylate synthetase. This phenomenon may in part account for the deviation from linearity seen in Fig. 6 at the lower deoxyuridine incorporation values.

The persistence of FdUMP in both ascites tumor cells and bone marrow for as long as 7 days after administration of 100 mg/kg of 5-FU was of interest; a similar persistence of FdUMP has been previously observed in L1210 solid tumor and murine bone marrow cells by Chadwick and Rogers (25). The present study suggests one possible explanation; dUMP may be expected to compete with FdUMP for the initial enzymes of the

pyrimidine catabolic pathway, 5′-nucleotidase and other phosphatases. The level of dUMP may have been sufficiently high so as to afford nearly complete protection of FdUMP from enzymatic breakdown in these tissues. Continued formation of stable FdUMP-enzyme complexes may occur and may help explain the gradual elimination of FdUMP observed. In addition, preferential loss of FdUMP-containing cells may occur by lysis or, in the case of the duodenal mucosa, by shedding into the intestinal lumen.

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