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Increased Levels of Thymidylate Synthetase in Cells Exposed to 5-Fluorouracil

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SUMMARY

The binding of 5-fluoro-2′-deoxyuridylate, methylenetetrahydrofolate, and thymidylate synthetase in a ternary complex results in enzyme inhibition and is a major component of 5-fluorouracil cytotoxicity in some cells. The amount of 5-fluoro-2′-deoxyuridylate bound to thymidylate synthetase in several human gastrointestinal tumor cell lines following 5-fluorouracil exposure was determined, using Sephadex G-25 chromatography and high-pressure chromatographic analysis. These data were compared with previously determined values for thymidylate synthetase levels in control cultures not exposed to 5-fluorouracil. In HuTu 80 cells, the amount of 5-fluoro-2′-deoxyuridylate bound to thymidylate synthetase represented 16% of the total amount of enzyme present in untreated cells. The values for 5-fluoro-2′-deoxyuridylate bound to thymidylate synthetase in the WIDR and HT 29 cell lines (57 and 46 fmoles/10^5 cells, respectively), however, exceed by 3- to 6-fold the total amount of this enzyme found in untreated cells. The presence of increased levels of thymidylate synthetase protein in these cell lines was confirmed by sodium dodecyl sulfate gel electrophoresis. Measurements of thymidylate synthetase levels following exposure of cells to cycloheximide demonstrated that thymidylate synthetase complexed to 5-fluoro-2′-deoxyuridylate has increased stability as compared with uncomplexed enzyme. The level of thymidylate synthetase (bound and free) present in WIDR cells was measured following removal of FUra from the media. After 48 hr, the level of bound enzyme had fallen from 53 to 14 fmoles/10^5 cells, whereas free enzyme, which was undectectable after a 24-hr exposure to FUra, returned to 60% of its level in untreated cells.

INTRODUCTION

The pyrimidine analogue FUra[1] is an antitumor agent which is widely used in the treatment of a variety of neoplasms. There are two mechanisms by which FUra, following its activation to nucleotide derivatives, is thought to exert its cytotoxic and chemotherapeutic effects: first, through inhibition of dTMP synthetase (EC 2.1.1.45) by the metabolite FdUMP, and second, by incorporation (at the level of FUTP) into cellular RNA (1). The former, which occurs through formation of a ternary complex containing the enzyme, FdUMP, and the CH_2FH_4 cofactor (2), results in depletion of cellular thymidylate pools and a cessation of DNA synthesis. FUra incorporation into RNA has been shown to affect RNA maturation and function (3, 4). The relative importance of these two mechanisms appears to vary among different cells and tumors (5, 6). In addition, recent reports have demonstrated the incorporation of FUra into DNA, and this phenomenon may also play a role in FUra cytotoxicity (7, 8).

Many studies have tried to correlate FUra cytotoxicity with either drug incorporation into RNA or dTMP synthetase inhibition (5, 6, 9, 10). Levels of RNA incorporation have been assessed by measurement of radioactivity in cellular RNA after treatment of cells with [^{3}H]FUra (5, 6, 10). The level of this incorporation often far exceeds the amount of [^{3}H]FdUMP found complexed to dTMP synthetase after such treatment. Because both RNA and the dTMP synthetase complex behave as acid-insoluble macromolecules, it has proved difficult to assess accurately the extent of complexation between [^{3}H]FdUMP and dTMP synthetase directly, although methods for such measurements have been described (11–13). Most often, the extent of dTMP synthetase inhibition following FUra treatment has been determined by measuring the amount of dTMP synthetase activity or

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[1] The abbreviations used are: dTMP synthetase, thymidylate synthetase; CH_2FH_4, 5,10-methylenetetrahydrofolate; FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FUrd, 5-fluorouridine; FUMP, 5-fluorouridine-5′-monophosphate; dTTP, 2′-deoxythymidine-5′-triphosphate; SDS, sodium dodecyl sulfate, dTMP synthetase:FdUMP:CH_2FH_4, the ternary complex formed between those three components; HPLC, high-pressure liquid chromatography.

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FdUMP binding capacity remaining in treated samples as compared with untreated controls (10, 14, 15). It has recently been demonstrated, however, that because of the reversible nature of the dTMP synthetase:FdUMP:CH_2FH_4 complex, these techniques can result in an overestimate of the amount of free dTMP synthetase present following FUra treatment (15).

We have been examining FUra metabolism in human gastrointestinal tumor cell lines using [6-^{3}H]FUra and have chosen to directly measure the amount of [^{3}H]FdUMP bound to dTMP synthetase following drug exposure.[2] The techniques employed take advantage of known stability characteristics of the complex formed between dTMP synthetase, FdUMP, and CH_2FH_4 (11), and allow accurate measurement of enzyme-bound [^{3}H]FdUMP in the presence of extensive incorporation of [^{3}H]FUra into RNA. Measurements of this type have led to the finding that dTMP synthetase levels are elevated in some cells following exposure to FUra. The details of this finding are the subject of this report.

MATERIALS AND METHODS

Chemicals. [6-^{3}H]FdUrd (specific activity, 18 Ci/mmole), [6-^{3}H]FUra (specific activity, 25 Ci/mmole), and [5-^{3}H]dUrd (specific activity, 22 Ci/mmole) were purchased from Moravek Biochemicals (City of Industry, Calif.). Tetrahydrofolic acid was prepared from folic acid (Sigma Chemical Company, St. Louis, Mo.) by the procedure of Hatefi *et al.* (16). Solutions of CH_2FH_4 (pH 9.5) were stored under vacuum at −80° in 0.12 M β-mercaptoethanol and 30 mM formaldehyde. The actual concentration of CH_2FH_4 was determined spectrophotometrically (17). L-(4,5)-[^{3}H]Leucine (specific activity, 45 Ci/mmole) and [2-^{14}C] thymidine (specific activity, 52 mCi/mmole) were purchased from Amersham (Arlington Heights, Ill.). All other chemicals were purchased from Sigma Chemical Company unless otherwise specified.

Cells and media. Human carcinoma cell lines were obtained from the following sources: HuTu 80 (18) and HT 29 (19), from the Naval Biosciences Laboratory (Berkeley, Calif.); WIDR (20), from Lederle Laboratories (Pearl River, N. Y.). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (KC Biologicals, Kansas City, Mo.) at 37° in an atmosphere of 92% air–8% CO_2. Doubling times for the cell lines under these conditions were as follows: HuTu 80, 21 hr; HT 29, 28 hr; and WIDR, 27 hr. Cells were monitored semimonthly for the absence of mycoplasma, using the Hoechst staining method (21).

Treatment of cells with [6-^{3}H]FUra. Cells (1×10^6) were seeded in 75-cm^2 tissue culture flasks (CoStar, Cambridge, Mass.) and allowed to attach and enter exponential growth (3 days, final density 1 to 3×10^5/cm^2). At this time, medium was replaced with fresh medium containing [6-^{3}H]FUra (25 Ci/mmole; final concentration 0.8 μM). The cells were incubated at 37° in a humidified 8% CO_2 atmosphere for 24 hr. Cells were harvested as previously described (22) and resuspended (1 to 2×10^7 cells/ml) in ice-cold 20 mM sodium phosphate (pH 7.3) containing 10 mM β-mercaptoethanol and 8 mM formaldehyde. The cell suspension was sonicated (Branson sonifier, setting 4, 3 sec), and cell debris was removed by centrifugation (4°, 15,000 × *g* for 5 min).

Isolation of [^{3}H]FdUMP bound in dTMP synthetase:FdUMP:CH_2FH_4 complex. Sonicate (0.2 ml) from cells treated with [6-^{3}H]FUra or from untreated cells in which a dTMP synthetase:[^{3}H]FdUMP:CH_2FH_4 complex had been formed by incubation with [6-^{3}H]FdUMP after sonication (22) was subjected to chromatography on a Sephadex G-25 column (1 × 30 cm) as previously described (11).

The void volume of the column (3–4 ml), containing the dTMP synthetase:FdUMP:CH_2FH_4 complex, was pooled and heated at 65° for 15 min to dissociate FdUMP from the complex (22). Following cooling and the addition of a known amount of [^{14}C]thymidine (5 μl, 50,000 dpm) to serve as an internal standard for recovery, the solution was brought to 5% in trichloroacetic acid by the addition of an appropriate volume of 50% ice-cold trichloroacetic acid, and the acid-precipitable material removed by centrifugation (4°, 1,000 × *g* for 5 min). The acidic supernatant was removed and added to an equal volume of cold Freon containing 0.5 M tri-*n*-octylamine according to the method of Khym (23). The mixture was vortexed and, after centrifugation (4°, 1,000 × *g* for 5 min), the aqueous upper phase was removed and concentrated using a Savant Speed Vac concentrator. The lyophilized sample was taken up in 0.1 ml of water containing 160 μM each of unlabeled FUra, FUrd, FdUrd, FUMP, and FdUMP and analyzed by HPLC as described below.

Acid-soluble metabolites of [^{3}H]FUra present in cell sonicates were extracted as described by Pogolotti *et al.* (12) and analyzed by HPLC in the presence of unlabeled standards, using the system described below.

Quantitation of [^{3}H]FdUMP bound in dTMP synthetase:FdUMP:CH_2FH_4 complex. Quantitation of [^{3}H]FdUMP isolated from the dTMP synthetase:FdUMP:CH_2FH_4 complex as described above was performed using an Altex gradient liquid chromatograph equipped with a fixed-wavelength (254 nm) UV detector. Separations were performed on a RP-C18 column (Alltech Associates, Deerfield, Ill.), using an ion-pairing gradient with 1 mM $(n\text{-Bu})_4N^+HSO_4^-$ and 5 mM potassium phosphate (pH 7) as the low-concentration eluent, and the same buffer, containing 40% methanol, as the high-concentration eluent. The elution consisted of an isocratic period of 5 min, followed by a 10-min linear gradient to a new isocratic period consisting of 25% high-concentration buffer, which was maintained for 32 min. At this time, a 15-min linear gradient to 100% high-concentration eluent was used to complete the run. The entire gradient was run at a flow rate of 2 ml/min. Fractions (1 ml) were collected directly into minivials and analyzed for radioactivity (on ^{3}H channel) by liquid scintillation counting. Such measurements were performed using 3a70b counting fluid (Research Products International, Elk Grove Village, Ill.) on a Beckman LS 6800 liquid scintillation counter. Those fractions containing [^{14}C]thymidine were recounted on a ^{14}C channel. The retention volumes for standards in this system are as follows: FUra, 9 ml; FUrd, 21 ml; FdUrd, 25 ml; Thd, 31 ml; FUMP, 52 ml; FdUMP, 63 ml. As noted above, the extracted samples were reconstituted in a solution containing unlabeled standards to allow accurate peak identification despite variability in retention volumes, which does occur during aging of the column.

For each sample, the amount of ^{3}H associated with the FdUMP standard was corrected for recovery during extraction and chromatography, as determined by the amount of [^{14}C]thymidine recovered. Recovery routinely fell between 65% and 72%.

Gel electrophoresis and autoradiography. Samples of cell sonicates to be compared were dissolved in electrophoresis sample buffer containing 75 mM Tris-sulfate, 2% SDS, 2% β-mercaptoethanol, 15% glycerol, and 0.001% bromophenol blue (pH 8.4) and heated at 100° for 10 min. Samples were analyzed by discontinuous polyacrylamide gel electrophoresis using the method of Maurer and Allen, as modified by Tegtmeyer *et al.* (24). The gels were fixed and stained with Coomasie blue according to the method of Fairbanks *et al.* (25), vacuum dried, and autoradiographed on Kodak Royal X-omat medical X-ray film. Autoradiograms were scanned with a Canalco Model K densitometer. Peaks were cut out and weighed to determine their relative areas.

dTMP synthetase activity following dialysis. Cell sonicates from control cells or from cells exposed to FUra (0.8 μM) or FdUrd (54 nM) were dialyzed against 100 volumes of 0.1 M sodium phosphate (pH 7) containing 10 mM β-mercaptoethanol, for up to 72 hr (with four changes of dialysis buffer). dTMP synthetase activity in the sonicates was assayed by the tritium displacement method of Roberts, as modified by Dolnick and Cheng (26).

Stability of intracellular dTMP synthetase and the dTMP synthetase:FdUMP:CH_2FH_4 complex. Cells (1×10^5) were plated in 60-cm^2

[2] W. L. Washtien, Comparison of 5-fluorouracil metabolism in two human gastrointestinal tumor cell lines. Manuscript in preparation.

tissue culture plates and allowed to attach and enter exponential growth. [6-^{3}H]FdUrd (18 Ci/mmole, final concentration, 54 nM) was added to one group of cells for 30 min to allow intracellular formation of a dTMP synthetase:[^{3}H]FdUMP:CH_2FH_4 complex (11). At the end of this time, the amount of complex present in these cells was determined by trichloroacetic acid precipitation onto glass-fiber filters. The amount of dTMP synthetase in extracts of untreated control cells was also determined at this time, using [6-^{3}H]FdUMP as an active-site titrant (22). Media on the remaining plates, half of which contained untreated controls and half of which were treated with [^{3}H]FdUrd, were replaced with fresh media containing cycloheximide (50 μg/ml). The amount of dTMP synthetase:[^{3}H]FdUMP:CH_2FH_4 complex in treated cells and of dTMP synthetase present in control cells were assayed as described above at selected times following the addition of cycloheximide.

Determination of free dTMP synthetase in cells exposed to [^{3}H]*FUra.* Sonicate (0.2 ml) from cells treated with [^{3}H]FUra was combined with 0.08 ml of a charcoal suspension (charcoal, 100 mg/ml; bovine serum albumin, 10 mg/ml; dextran, 0.5 mg/ml) to remove endogenous nucleotides (15). After centrifugation to remove the charcoal, free dTMP synthetase was complexed to [^{3}H]FdUMP by incubation with this compound in the presence of CH_2FH_4 (22). The amount of [^{3}H]FdUMP bound to dTMP synthetase was then determined as described above. The amount of free dTMP synthetase was calculated by subtracting the amount of [^{3}H]FdUMP found bound to dTMP synthetase in sonicates which were not incubated with [6-^{3}H]FdUMP from the values obtained for [^{3}H]FdUMP bound to dTMP synthetase in sonicates which had been incubated with [6-^{3}H]FdUMP.

RESULTS

[^{3}H]*FdUMP bound to dTMP synthetase following exposure of cells to [6-^{3}H]FUra.* The amount of [^{3}H]FdUMP bound to dTMP synthetase in several cell lines exposed to [6-^{3}H]FUra (0.8 μM, 24 hr) was determined using Sephadex G-25 chromatography and HPLC. The results are shown in Table 1. Table 1 also includes the level of dTMP synthetase which we had previously determined to exist in these cell lines, utilizing direct titration of the enzyme from untreated cells with [6-^{3}H]FdUMP (22).

The HuTu 80 cell line has only 16% of its total dTMP synthetase bound to [^{3}H]FdUMP. In the WIDR and HT 29 cells, however, the amount of [^{3}H]FdUMP bound to this enzyme is 3- to 6-fold greater than the previously determined level of dTMP synthetase for these cells. The same phenomenon, i.e., levels of FdUMP bound to dTMP synthetase in excess of control values of the enzyme, was observed following treatment of WIDR cells with [6-^{3}H]FdUrd.

TABLE 1

[^{3}H]FdUMP bound to dTMP synthetase

[^{3}H]FdUMP bound to dTMP synthetase in cells exposed to [6-^{3}H]FUra (0.8 μM, 24 hr) was determined as described under Materials and Methods.

Cell line	[^{3}H]FdUMP bound	Free [^{3}H]FdUMP[a]	dTMP synthetase[b]
	fmoles/10^5 cells		
HuTu 80	12[c]	1[d]	72
HT 29	46	69	7.2
WIDR	57	42	17.6

[a] Determined as described under Materials and Methods.

[b] Previously determined by titration with [^{3}H]FdUMP (22). Titrations of this nature were repeated to ensure that no changes in the enzyme level of untreated cells had occurred since the initial experiments were performed. The same values were obtained.

[c] Each value was the mean of duplicate experiments; the standard error of the mean was less than 10% of the mean value in all cases.

[d] Lower limit of detection, 1 fmole/10^5 cells.

One can also measure the amount of free FdUMP generated in each of these cell lines following FUra exposure. As shown in Table 1, no detectable free FdUMP is found in HuTu 80 cells, suggesting that all of the FdUMP generated in these cells is bound to dTMP synthetase. In both HT 29 and WIDR cells, however, free FdUMP could be detected. Although the data are not shown, free FdUMP is also generated following treatment of WIDR cells with FdUrd.

The method we have employed to measure [^{3}H]FdUMP bound to dTMP synthetase in cells following exposure to [6-^{3}H]FUra takes advantage of known stability properties of the complex formed between dTMP synthetase, FdUMP, and CH_2FH_4 (11), i.e., stability to gel chromatography and acid precipitation, lability to heating at 65° for 15 min. The values obtained for [^{3}H]FdUMP bound to dTMP synthetase using this method, however, exceeded the expected limiting value (i.e., the total cellular dTMP synthetase as measured by titration with FdUMP) in both cell lines which also contained free [^{3}H]FdUMP. We therefore investigated whether our measurements included intracellular [^{3}H]FdUMP not associated with dTMP synthetase.

To test this possibility, a sonicate of WIDR cells exposed to [6-^{3}H]FUra was chromatographed on Sephadex G-25, and the excluded volume was pooled. Figure 1A shows the HPLC analysis of acid-soluble material present in this sample. Almost no radioactivity is found in this fraction; the small amount of tritium associated with FdUMP corresponds to only 0.4 fmole/10^5 cells. If the material excluded from Sephadex G-25 is heated prior to extraction of acid-soluble material, HPLC analysis (Fig. 1B) reveals one main peak of tritium, which coelutes with authentic FdUMP and equals 64 fmoles/10^5 cells. The nonspecific background of ^{3}H associated with FdUMP in unheated samples is less than 1% of the value obtained in the heated sample. This result indicates that the levels of [^{3}H]FdUMP associated with dTMP synthetase which we have measured in cells exposed to [6-^{3}H]FUra are not artificially increased by either free [^{3}H]FdUMP, which has not been adequately separated from macromolecular-bound material by gel chromatography, or by loosely bound [^{3}H]FdUMP, which can be dissociated merely by treatment with acid.

Furthermore, when these methods (gel chromatography, heating, HPLC) are utilized to redetermine the level of dTMP synthetase in WIDR cells following direct titration of the enzyme in a cell sonicate with [^{3}H]FdUMP (22), the value obtained (16 fmoles/10^5 cells) is the same as that previously measured using trichloroacetic acid precipitation (see Table 1).

Direct evidence for increased dTMP synthetase in fluoropyrimidine-treated cells. The experiments described above indicate that the higher-than-expected values obtained for [^{3}H]FdUMP bound to dTMP synthetase in some cells exposed to [6-^{3}H]FUra represent a drug-related increase in the amount of cellular enzyme.

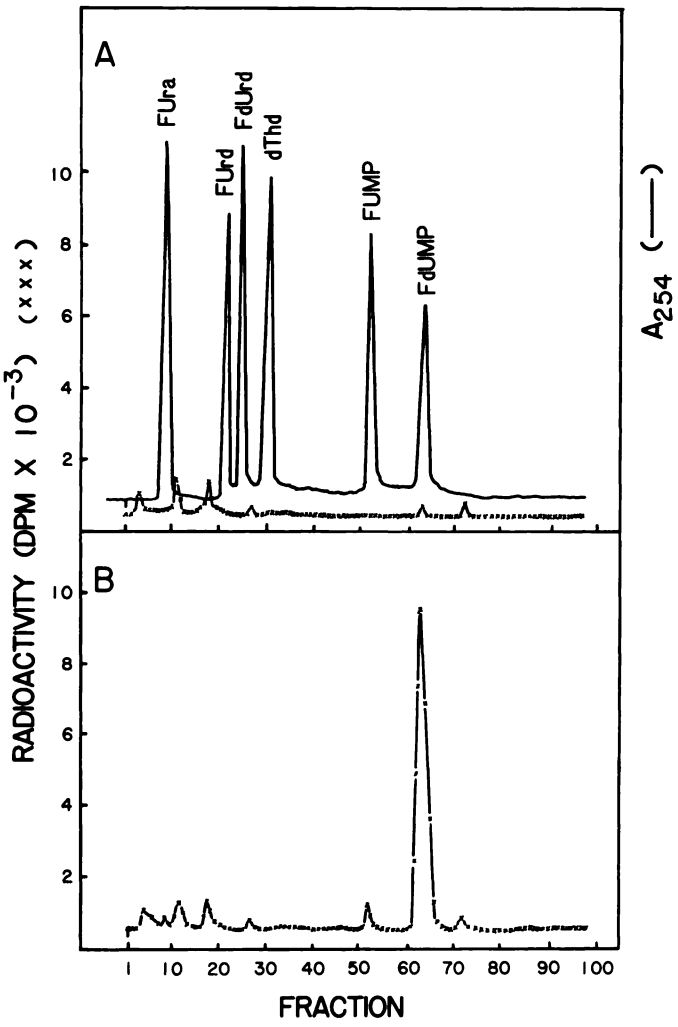

FIG. 1. *HPLC analysis of acid-soluble radioactivity complexed to macromolecules following Sephadex G-25 chromatography*

A sonicate of WIDR cells which had been exposed to [6-^{3}H]FUra was chromatographed on Sephadex G-25 as described under Materials and Methods. The excluded volume was pooled, [^{14}C]thymidine was added, and the sample was divided into two equal parts. Acid-soluble material was extracted as described under Materials and Methods either (A) without further treatment or (B) after heating at 65° for 15 min. The extracted material was then analyzed using HPLC. The unbroken line (——) represents the UV absorbance of unlabeled standards. The dashed line (×——×) represents the ^{3}H present in extracted material. The recovery in both samples, based on [^{14}C]thymidine recovery, was 70%.

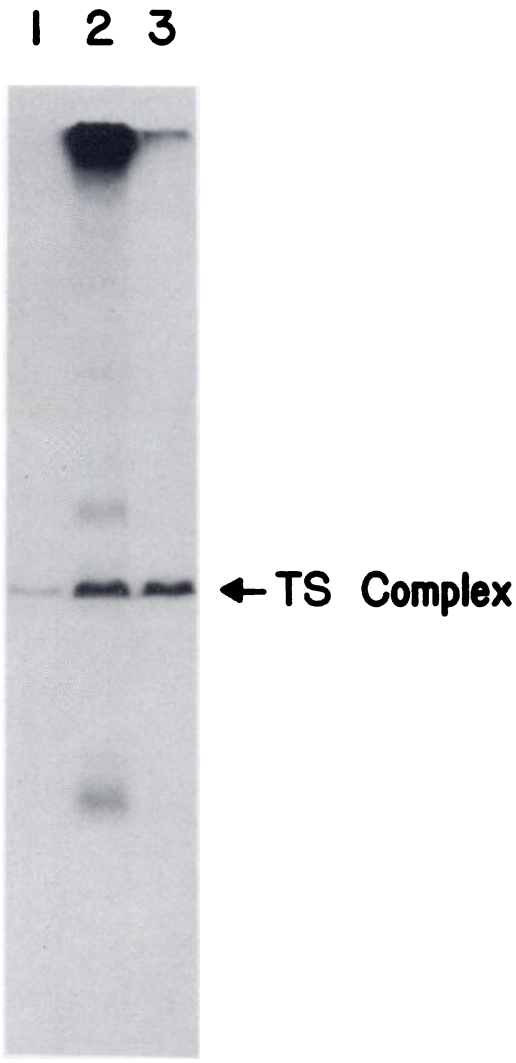

FIG. 2. *dTMP synthetase:FdUMP:CH_2FH_4 complex present in untreated and fluoropyrimidine-treated WIDR cells*

Equal amounts of protein from WIDR cells which were untreated (Lane *1*) or expoed to [6-^{3}H]FUra (Lane *2*) or [6-^{3}H]FdUrd (Lane *3*) were subjected to SDS gel electrophoresis as described under Materials and Methods. A gel autoradiogram is shown. The dTMP synthetase:FdUMP:CH_2FH_4 complex was formed in untreated cells by incubation of cell sonicate with [^{3}H]FdUMP (22) prior to electrophoresis.

The dTMP synthetase:FdUMP:CH_2FH_4 complex has been shown to be stable to SDS gel electrophoresis, the complex traveling as one band with a molecular weight of 34,000 (27). We utilized this technique to demonstrate that the increase in dTMP synthetase-bound [^{3}H] FdUMP is reflected by an increase in the amount of complex (i.e., protein) present in this band.

Figure 2 is an autoradiogram of an SDS gel of cell sonicates from both untreated WIDR cells (in which [6-^{3}H]FdUMP was used to titrate dTMP synthetase directly) and from WIDR cells exposed to either [6-^{3}H]FUra or [6-^{3}H]FdUrd for 24 hr. Equal amounts of protein were placed in each lane. The results clearly demonstrate an increase in the amount of protein migrating as the dTMP synthetase:FdUMP:CH_2FH_4 complex in treated versus control cells. Table 2 contains the integrated areas for each sample and the amount of [^{3}H] FdUMP bound to dTMP synthetase measured in the same sample by the G-25, HPLC method. The relative areas correlate well with the measured amount of dTMP synthetase-bound [^{3}H]FdUMP, and provide further evidence for a drug-mediated increase in cellular dTMP synthetase.

Intracellular stability of dTMP synthetase. There have been several reports in which an increase in the intracellular activity or content of a target enzyme has been observed following exposure of cells or tissues to a drug which is known to complex to that enzyme (28, 29). In these cases, altered enzyme levels were found to be due

TABLE 2

[^{3}H]FdUMP bound to dTMP synthetase: direct measurement versus relative intensity on gels

The autoradiogram shown in Fig. 2 was scanned on a Canalco densitometer, and the areas under the peaks were determined as described under Materials and Methods. The amount of [^{3}H]FdUMP bound to dTMP synthetase in each sample was determined as described under Materials and Methods.

Lane[a]	Relative integrated area[b]	[^{3}H]FdUMP bound
		fmoles/10^5 cells
1	1	15
2	4.5[c]	56
3	5.7	57

[a] See Fig. 2 for the content of each lane.

[b] The smallest area was given an arbitrary value of 1.

[c] Corrected for the difference in specific activity between the [6-^{3}H]FUra and [6-^{3}H]FdUrd, [^{3}H]FdUMP used in the experiments.

to *in vivo* stabilization of the enzyme by its drug inhibitor. We therefore examined the relative *in vivo* stability of dTMP synthetase and [^{3}H]FdUMP-bound dTMP synthetase.

Figure 3 demonstrates the influence of [^{3}H]FdUMP binding on the *in vivo* stability of dTMP synthetase. When protein synthesis is inhibited by treatment with cycloheximide, the level of dTMP synthetase in control cells declined with a $t_{1/2}$ of approximately 6 hr. In cells pretreated with [6-^{3}H]FdUrd, so that dTMP synthetase had been complexed to [^{3}H]FdUMP prior to exposure to cycloheximide, the amounts of [^{3}H]FdUMP-bound dTMP synthetase remaining after 16 hr represented 75% of the original value. These data clearly demonstrate an increased stability of dTMP synthetase bound to FdUMP as compared with uncomplexed enzyme.

Recovery of dTMP synthetase activity. The experiments thus far presented demonstrate that an increased level of dTMP synthetase present as a dTMP synthetase:FdUMP:CH_2FH_4 complex exists after exposure of some cells to [6-^{3}H]FUra. This complex, although stable, is known to be reversible (2). We therefore examined the *in vitro* and *in vivo* reversibility of the complex formed within WIDR cells following FUra exposure. Sonicates from control and FUra-treated WIDR cells were dialyzed for 72 hr to remove enzyme-bound FdUMP. Table 3 contains the dTMP synthetase activity found in each sonicate following dialysis. The activity in the treated cells is 200–250% of that found in sonicates of untreated cells.

It is known that reversal of FdUMP-mediated dTMP synthetase inhibition can occur within cells. We measured the amount of *both* [^{3}H]FdUMP-bound and free dTMP synthetase during and following exposure of WIDR cells to [6-^{3}H]FUra. Table 4 presents the results of this experiment. The level of [^{3}H]FdUMP bound to dTMP synthetase rises during exposure to [^{3}H]FUra, reaching the value previously observed at 24 hr of exposure. In addition, free intracellular [^{3}H]FdUMP is present. Twenty-four hours after removal of the drug, the amount of [^{3}H]FdUMP bound enzyme has decreased, and free dTMP synthetase is once more observed in the cell. Free intracellular [^{3}H]FdUMP is no longer detected.

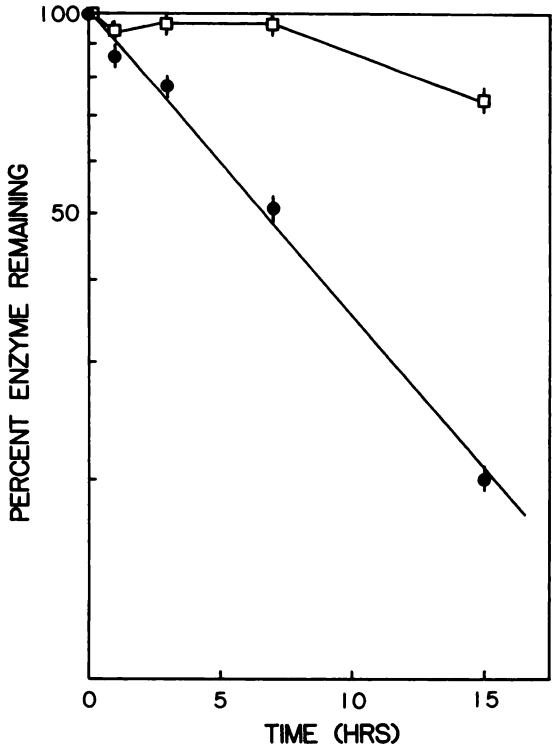

FIG. 3. *In vivo stability of unbound and FdUMP-bound dTMP synthetase*

The level of unbound dTMP synthetase and dTMP synthetase complexed to FdUMP in WIDR cells following the addition of cycloheximide (50 μg/ml) was determined as described under Materials and Methods. The amount of enzyme present at the times indicated is expressed as a percentage of the value determined at 0 time. Each point represents the mean value of three experiments. *Vertical lines* indicate standard error of the mean. At each point, incorporation of [^{3}H]leucine into acid-soluble material was measured, and indicated that 95% inhibition of protein synthesis was maintained during the entire experiment. Unbound dTMP synthetase is represented by ●; FdUMP-bound dTMP synthetase is represented by □.

DISCUSSION

We have examined the extent of binding of [^{3}H]FdUMP to dTMP synthetase in three human gastrointestinal tumor cell lines, following exposure of the cells to [6-^{3}H]FUra. A comparison of the values obtained from these measurements with the levels of dTMP synthetase previously determined in untreated cells has demonstrated that FUra exposure can result in an increase in the level of intracellular dTMP synthetase. This conclusion is based on the amount of [^{3}H]FdUMP bound to the

TABLE 3

dTMP synthetase activity following dialysis

Sonicates of control and fluoropyrimidine-treated WIDR cells were dialyzed against 0.1 M sodium phosphate (pH 7) for 72 hr. dTMP synthetase activity following dialysis was determined as described under Materials and Methods.

Sample	dTMP synthetase activity
	pmoles/mg/min
Untreated cells	4.3
FUra exposure for 24 hr	11.2
FdUrd exposure for 24 hr	11.5

TABLE 4

[^{3}H]FdUMP-bound and free dTMP synthetase in WIDR cells exposed to [6-^{3}H]FUra

WIDR cells were exposed to [6-^{3}H]FUra (0.4 μM) for 24 hr. At 4 and 24 hr after addition of drug, the amount of [^{3}H]FdUMP-bound dTMP synthetase, free dTMP synthetase, and free [^{3}H]FdUMP in the cells was determined as described under Materials and Methods. At 24 hr, cells were placed in drug-free medium and the same parameters were measured 24 and 48 hr after the medium change.

Time	[^{3}H]FdUMP dTMP synthetase	Free dTMP synthetase	Free [^{3}H]FdUMP
		fmoles/10^5 cells	
In presence of drug			
0 hr	0	17	0
4 hr	2.1	14.8	1
24 hr	53.2	0	10
After drug removal			
24 hr	34	10	1
48 hr	14	10	1

enzyme and the fact that the number of FdUMP binding sites can be taken as a direct measure of the enzyme level in cells (14). The presence of increased amounts of dTMP synthetase in FUra-exposed cells could also be demonstrated directly by a comparison of the amount of material migrating as the dTMP synthetase: FdUMP:CH_4FH_4 complex in SDS gel electrophoresis of samples from treated and control cells.

The increase in total cellular dTMP synthetase (although bound to FdUMP) which we observed appears to be due to an increase in the *in vivo* stability of dTMP synthetase complexed to FdUMP as compared with uncomplexed dTMP synthetase. Examination of *in vivo* levels of dTMP synthetase and FdUMP-bound dTMP synthetase following exposure of cells to cycloheximide clearly illustrates an increased lifetime within cells for the complexed enzyme. These results are similar to previous reports of stabilization (and resultant increases in total enzyme) of dihydrofolate reductase by methotrexate and *S*-adenosylmethionine decarboxylase by methylglyoxal bis(guanylhydrazone) (28–30).

It is possible that, in addition, the rate of synthesis of dTMP synthetase is increased in response to changes in metabolite pools which result from dTMP synthetase inhibition, most significantly the depletion of dTTP. Two factors argue against enzyme synthesis as an important determinant in the observed increase in dTMP synthetase. First, the half-time for turnover of dTMP synthetase (6 hr) is slow in comparison to the observed turnover of enzymes, such as ornithine decarboxylase, whose rate of synthesis is known to be an important site of metabolic regulation (31). Second, if one exposes WIDR cells to [^{3}H]FdUrd in the presence of thymidine, which will prevent dTTP depletion, the value obtained for [^{3}H]FdUMP bound to dTMP synthetase is similar to that observed in the absence of thymidine. The differences observed in the percentage increase of dTMP synthetase in WIDR versus HT 29 cells most likely reflect differences in the basal rate of synthesis of dTMP synthetase in the two cell lines.

Our results suggest that increased levels of cellular dTMP synthetase arise in cells which generate intracellular FdUMP sufficient to both bind the enzyme initially present in the cells, and to then titrate newly synthesized enzyme molecules. This is the situation which occurs in both WIDR and HT 29 cells during exposure to 0.8 μM [6-^{3}H]FUra (and to WIDR cells exposed to FdUrd). HuTu 80 cells are unable to generate sufficient FdUMP when exposed to this concentration of FUra to bind all of the enzyme present in the cells (12 fmoles/10^5 cells FdUMP generated versus 72 fmoles/10^5 cells dTMP synthetase), and no accumulation of dTMP synthetase is seen. However, when HuTu 80 cells are exposed to higher concentrations of FUra, we have observed a 300% increase in intracellular dTMP synthetase levels.[3]

Complexation of FdUMP to dTMP synthetase requires CH_2FH_4, and it has been shown that CH_2FH_4 can be limiting to complex formation even when FdUMP levels are adequate (9, 27). We have previously shown that, for the cells examined in this paper, endogenous folate levels are sufficient to allow maximal binding of [^{3}H]FdUMP to dTMP synthetase in untreated control cells (22). It is possible, however, that continued binding of newly synthesized dTMP synthetase could be compromised by insufficient folate pools. If this occurred, one would detect both free FdUMP and unbound dTMP synthetase in the cells simultaneously, unable to form a ternary complex because of lack of folate. For the one cell line in which such measurements have been performed (Table 4), this situation does not arise. Free FdUMP and unbound dTMP synthetase are not detected simultaneously at any time during or following drug exposure.

The implications of these results for FUra cytotoxicity mediated through dTMP synthetase inhibition appear to be 2-fold. First, it is likely that FdUMP levels in excess of the initial level of cellular dTMP synthetase will be required to maintain an inhibition of this enzyme. Because dTMP synthetase levels vary subtantially among cells (22), achievement of cytotoxic levels of FdUMP may be impossible in some cells. The cytotoxicity of FUra in these cases would most likely be related to RNA effects of the drug. Second, even in cells in which initial dTMP synthetase levels are not high and FdUMP synthesis is adequate, sustained dTMP synthetase inhibition may be possible only with prolonged exposure to drug, as occurs during infusion therapy. Within 24 hr of removal of FUra from the media, WIDR cells had regained 59% of the original level of dTMP synthetase in an unbound form and no longer contained detectable free FdUMP. Continued exposure of such cells to FUra might allow an excess of FdUMP to exist long enough for dTMP synthetase inhibition to be cytotoxic.

Finally, the method we have used allows us to monitor directly the interaction of intracellular [^{3}H]FdUMP with dTMP synthetase following exposure of cells to [^{3}H]FUra. We believe that this approach is useful in better understanding mechanisms of FUra cytotoxicity in cells, and studies utilizing this methodology are ongoing in our laboratory.

[3] W. L. Washtien, unpublished observations.

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