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Nucleotide Levels and Incorporation of 5-Fluorouracil and Uracil into DNA of Cells Treated with 5-Fluorodeoxyuridine

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SUMMARY

Intracellular pools of 5-fluoro-2′-deoxyuridine (FdUrd) and dUrd nucleotides were measured in cultured human lymphoblasts treated with FdUrd. At 1 μM FdUrd, intracellular 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) was ~400 pmoles/10^6 cells, and FdUTP was ~0.1 pmole/10^6 cells. Intracellular dUMP and dUTP were elevated to values of ~1000 pmoles/10^6 cells and ~0.1 pmole/10^6 cells, respectively. With decrease in dTTP levels, utilization of FdUTP and dUTP as substrates for DNA synthesis became significant. FdUMP and dUMP, ~5 pmoles of each per micromole of DNA nucleotide, were found in DNA of cells treated with FdUrd (1 μM). The active removal of FUra and Ura from DNA of FdUrd-treated cells by the normal repair mechanism may lead to fragmentation of DNA and contribute to the cytotoxic effect of FdUrd.

INTRODUCTION

The block in DNA synthesis that results from treatment of cells with FUra[2] or FdUrd is accounted for by inhibition of thymidylate synthetase by the intracellular monophosphate derivative, FdUMP (1). The corresponding di- and triphosphates of FdUrd have not been identified in cells treated with either of these drugs, although the pathway by which dTTP is formed is capable of utilizing FdUMP as well. An explanation for the apparent absence of intracellular FdUTP has been provided by the demonstration that FdUTP is a substrate for the enzyme, dUTPase (2, 3). This, together with the ability of Ura-DNA glycosylase to remove FUra from DNA (2, 3), also accounts for the inability to demonstrate FUra in DNA (1). It has been shown previously that inhibition of thymidylate synthetase activity by methotrexate, which acts by limiting availability of reduced folate, is accompanied by elevation of dUTP (4).

In studies described here, the intracellular nucleotides of FdUrd and dUrd, as well as the uptake of FUra and Ura into DNA, were analyzed in cells treated with FdUrd.

MATERIALS AND METHODS

Cell labeling. All experiments were carried out using cultured human lymphoblasts (8866) grown in modified Eagle's medium as described previously (4). Cells were treated with FdUrd at either 0.1 or 1 μM under growth conditions for a total of 6 hr. During the last 2 hr of exposure to FdUrd, the cells were labeled with either [^{3}H]FdUrd or [^{3}H]dUrd.

For labeling of DNA, [6-^{3}H]FdUrd (15 Ci/mmole) (Moravek) was added at 0.1 and 0.8 μM for cells in 0.1 and 1 μM FdUrd, respectively. For labeling of cellular nucleotide pools, [^{3}H]FdUrd was at 0.1 μM for both levels of FdUrd in the medium, and additional unlabeled FdUrd was added with the higher level of [^{3}H]FdUrd, giving final concentrations of 0.2 and 1.8 μM, respectively.

For both levels of FdUrd in the medium, [6-^{3}H]dUrd (25 Ci/mmole) was used at 0.1 μM to label nucleotide pools, and [5-^{3}H]dUrd (15 Ci/mmole) was used at 1 μM for labeling of DNA.

Cells not treated with FdUrd were labeled with 0.1 μM [6-^{3}H]dUrd for 30 min.

Determination of intracellular dUTP. The procedure used to measure dUTP was modified from one used previously (4) by the substitution of a column chromatography step for two paper chromatography steps, and the inclusion of an IO_4^- oxidation step to destroy ribonucleotides. The previous report (4) should be consulted for details of the subsequent steps and other procedures described below. Briefly stated, cells (100 ml at ~8×10^5/ml) labeled with [^{3}H]dUrd were rapidly harvested by centrifugation and extracted with trichloroacetic acid containing 100 nmoles of dUTP as internal standard, followed by removal of the acid by solvent extraction. The extract was applied to a column of DEAE-Sephadex (Pharmacia, A-25, 1-ml bed volume). This was eluted with 20 ml of 2 M HCOOH/0.2 M NH_4COOH to remove NMPs, most NDPs, CTP, and ATP and then washed

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[2] The abbreviations used are: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; FdUTP, 5-fluoro-2′-deoxyuridine 5′-triphosphate; FdUDP, 5-fluoro-2′-deoxyuridine 5′-diphosphate; HPLC, high-pressure liquid chromatography.

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with 15 ml of water, followed by a 40-ml linear gradient of triethylammonium bicarbonate (pH 8.5), 0.02–0.5 M. Fractions were pooled according to the expected position for dUTP relative to the rGTP peak, as determined by experiments with markers.

The pooled fractions containing dUTP were dried, redissolved in 0.1 ml of water, treated with IO_4^-/methylamine (5), and then chromatographed (5½ d) on paper (Schleicher & Schuell 589 Orange Ribbon-C) in isobutyric acid/H_2O/NH_4OH, 66:33:1 (v/v/v) (Fig. 1A). The dUTP region, localized by internal or external markers, was eluted, dephosphorylated with bacterial alkaline phosphatase, and analyzed for absorbance (A_{254}) and radioactivity by HPLC with a C-18 reverse-phase column (Lichrosorb, 4.6 × 250 mm) using 0.02 M potassium phosphate (pH 3.7) at a flow rate of 1.5 ml/min. Fractions (0.3 ml) were collected and counted in Triton X-100-toluene scintillation fluid (Fig. 1B). From the amount of dUrd (A_{254}), representing recovery of the dUTP internal standard, the radioactivity in dUrd was corrected for losses incurred during the isolation procedure. The corrected radioactivity plus specific activity (see below) gave the amount of dUTP in the original acid extract of cells.

Alternatively, in some experiments the IO_4^-/methylamine step was carried out prior to the DEAE-Sephadex chromatography and the fractions were pooled on the basis of absorbance of the dUTP internal standard. The results for the two procedures were the same.

Determination of intracellular FdUTP. The procedure used to measure FdUTP was similar to that for dUTP, described above. To isolate FdUTP, acid extracts of [^{3}H]FdUrd-labeled cells containing internal standard of FdUTP (25 nmoles) [prepared from FdUrd by the methods of Yoshikawa *et al.* (6) and Sowa *et al.* (7)] were chromatographed on a column of DEAE-Sephadex (see above), oxidized with IO_4^-, and chromatographed on paper in ammonium isobutyrate (Fig. 2A).

FdUrd derived from FdUTP by phosphatase treatment was analyzed by HPLC on a C-18 reverse-phase column (Fig. 2B), and the associated radioactivity, corrected for losses using the recovery of internal standard, together with specific activity, gave the value for intracellular FdUTP.

Analysis of DNA for dUMP and FdUMP. FdUrd-treated cells (20 ml) labeled with [^{3}H]dUrd (or [^{3}H]FdUrd) were analyzed to determine the amount of [^{3}H]dUMP (or [^{3}H]FdUMP) in DNA using a procedure described previously (8). The DNA from the cells was freed of protein, RNA, and low molecular weight contaminants (including nucleosides and nucleotides); the DNA was digested to dNMPs, which were chromatographed with carrier dUMP (and/or FdUMP) on paper in ammonium isobutyrate. The dUMP (or FdUMP) was dephosphorylated with phosphatase, and the deoxynucleoside was isolated by HPLC on a C-18 reverse-phase column (Fig. 3). The radioactivity in dUrd or FdUrd was related to amount of DNA from the associated dGuo, which copurified (as dGMP) with dUMP and FdUMP in the paper chromatography step. Radioactivity was converted to amount with the specific activities (see below).

Determinations of specific activities. The specific activities of nucleotides in FdUrd-treated cells labeled with [^{3}H]dUrd were determined by a procedure described previously (4). Briefly stated, an acid extract was prepared as described above for determination of intracellular dUTP, but without the internal standard of dUTP. dUMP was isolated by paper chromatography in ammonium isobutyrate and converted to dUrd with phosphatase; it was then purified by paper chromatography in 1-butanol/NH_4OH and analyzed for amount (A_{254}) and radioactivity by HPLC on a C-18 reverse-phase column. The value for dUMP in cells labeled with [^{3}H]dUrd in 0.1 and 1 μM FdUrd was 141 cpm/pmole. The specific activities of the di- and triphosphates of dUrd were assumed to be the same as for dUMP.

For specific activity of dUrd nucleotides in [^{3}H]dUrd-labeled cells not treated with FdUrd, the procedure was the same as above except that dTTP, rather than dUMP, was purified and analyzed. This gave a value of 470 cpm/pmole for dTTP (and dUrd nucleotides).

Specific activity of FdUMP in [^{3}H]FdUrd-labeled cells determined by the procedure described above did not differ from that measured directly on the [^{3}H]FdUrd used to label the cells.

Determination of other nucleotides. Mono- and diphosphates of dUrd or FdUrd in [^{3}H]dUrd- or [^{3}H]FdUrd-labeled cells were determined by using the specific activity to convert total counts in the trichloroacetic acid extract to amount of nucleotide, and analyzing for distribution between mono- and diphosphate by paper chromatography in ammonium isobutyrate. In some cases (i.e., for cells not treated with FdUrd), additional steps were used to resolve dUMP and dUDP from dTMP and dTDP. This was accomplished by elution, dephosphorylation with phosphatase, and rechromatography on paper in 1-butanol/NH_4OH (4).

Levels of dTTP were determined as the nucleotide after separation from dTMP and dTDP. Extracts of unlabeled cells were prepared with trichloroacetic acid to which a small amount of [^{3}H]dTTP had been added. The acid was removed, and the sample was treated with IO_4^- and then chromatographed on a column of DEAE-Sephadex (see above), using the radioactivity to identify fractions containing dTTP. The pooled fractions were dried and treated with phosphatase, and the amount of dTTP was measured by HPLC (A_{254}) with a C-18 reverse-phase column (see above), using recovery of ^{3}H to correct for losses.

RESULTS

Intracellular dUTP and FdUTP. Most of the intracellular nucleotide measurements in these studies were made on cells in which the cellular pools were labeled with radioactive nucleoside in growth medium. The labeling period was sufficiently long to allow equilibration and to approach steady-state labeling conditions (4). Under the conditions used in the experiments described here, there was no greater than 10–15% uptake of [^{3}H]dUrd or [^{3}H]FdUrd, and no evidence of interference of FdUrd with uptake of [^{3}H]dUrd or equilibration of nucleotide pools (data not shown).

Treatment of cells with FdUrd caused a striking increase in intracellular dUMP as measured by chromatographic distribution of [^{3}H]dUrd-labeled intracellular nucleotides and specific activity (Table 1).

dUTP was isolated by successive ion exchange and

TABLE 1

Intracellular dThd, dUrd, and FdUrd Nucleotides[a]

FdUrd in culture medium[b]	dTTP	dUMP	dUDP	dUTP	FdUMP	FdUDP	FdUTP
μM	*pmoles/10⁶ cells*						
0	48	1.6	0.2	—[c]			
0.1	11	1021	8.4	0.074	48	0.15	0.023
1.0	0.8	1080	3.6	0.055	431	0.60	0.12

[a] Each value represents the average of at least two determinations.

[b] For the measurements of cellular FdUMP, FdUDP, and FdUTP, the concentration of FdUrd in the medium was increased from 0.1 and 1.0 μM to 0.2 and 1.8 μM, respectively, during the last 2 hr of the 6-hr exposure to FdUrd.

[c] Previously reported attempts to measure dUTP in these cells indicated a value ≤0.3 fmole/10⁶ cells.

paper chromatography (Fig. 1A) and, after dephosphorylation, was measured as dUrd by HPLC on a reverse-phase column (Fig. 1B) (see Materials and Methods). The value for radioactivity in dUTP, corrected for losses and combined with independently measured specific activities, gave the amount of dUTP. Intracellular dUTP is increased, from the previously determined value of ≤0.3 fmoles/10⁶ cells without drug treatment (4), to 0.05–0.07 pmoles/10⁶ cells when treated with FdUrd at 0.1 or 1.0 μM (Table 1).

Measurements of FdUrd nucleotides were carried out with procedures similar to those described for dUrd nucleotides (Fig. 2). With a 10-fold increase of FdUrd in the medium, intracellular levels of FdUrd nucleotides showed a corresponding 5- to 10-fold increase, and, at the higher FdUrd in the medium (1 μM) intracellular FdUMP and FdUTP were close to the levels of dUMP and dUTP, respectively (Table 1). In contrast to the increase in FdUrd nucleotides with increase in FdUrd in the culture medium, intracellular dUMP remained approximately the same, and dUDP and dUTP may even have de-

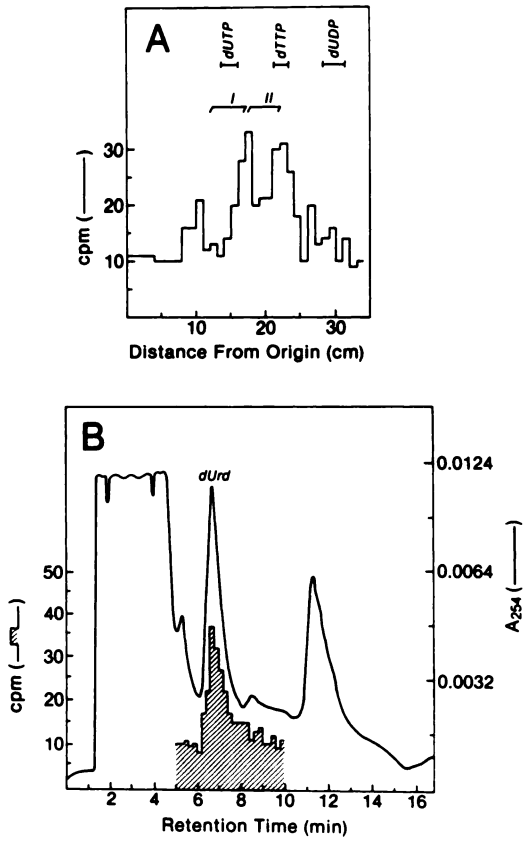

FIG. 1. *Purification and HPLC analysis of intracellular dUTP*

Acid extract was prepared from FdUrd-treated cells (1 μM) labeled with [³H]dUrd, and was fractionated on a DEAE-Sephadex column (see Materials and Methods). Pooled fractions containing dUTP were chromatographed on paper in ammonium isobutyrate (A). Positions of outside markers are indicated at *top*. Fractions pooled as indicated (*brackets*) were dephosphorylated with phosphatase and analyzed by HPLC on a reverse-phase column (B) (Pool 1 from A shown here). The effluent was analyzed for recovery of internal standard (A_{254}) and radioactivity in [³H]dUrd (from [³H]dUTP) (see Materials and Methods).

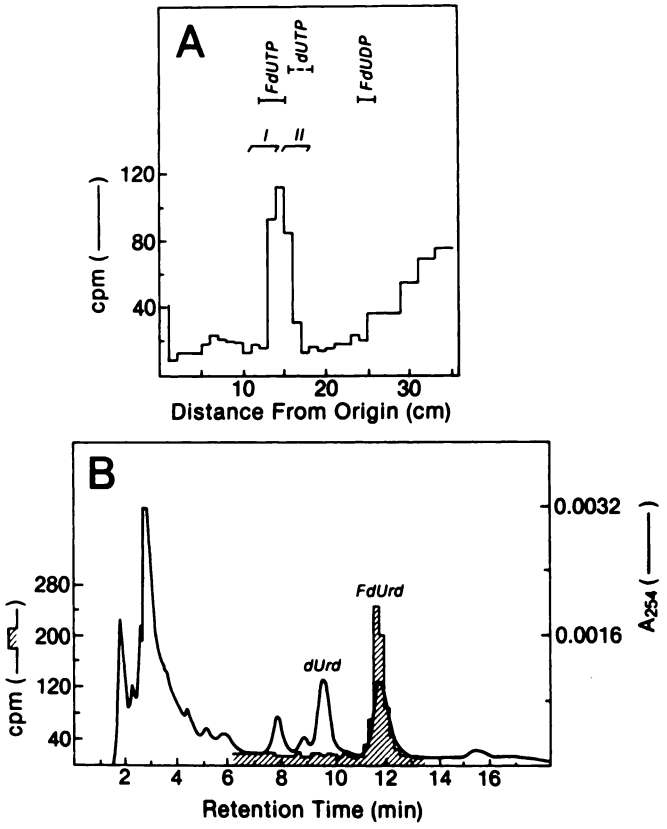

FIG. 2. *Purification and HPLC analysis of intracellular FdUTP*

The acid extract of FdUrd-treated cells (1 μM) labeled with [³H]FdUrd, purified by DEAE-Sephadex column chromatography, was further processed as in Figure 1 by paper chromatography (A) followed by HPLC (B) (Pool 1 from A shown here). (Position of dUTP marker in A was based on relative R_F determined in separate chromatograms.) The A_{254} peak of FdUrd in B resulted from the FdUTP internal standard (included with acid extraction); dUrd was added as a marker prior to HPLC.

creased somewhat with the higher levels of inhibitor (see Discussion).

Ura and FUra in DNA. Both dUTP and FdUTP are utilized by DNA polymerases and, with the fall in intracellular dTTP due to the inhibitory effect of FdUMP on thymidylate synthetase, dUTP and FdUTP may become significant as substrates for synthesis of DNA. Although there is a mechanism for removal of Ura and FUra from DNA (2, 3, 9), under these circumstances it is possible that residual amounts of Ura and/or FUra may be detected in DNA (8).

To test for uptake and retention of Ura in DNA, cells were treated with FdUrd and labeled with [^{3}H]dUrd. DNA was isolated free of nucleosides or nucleotides and digested to dNMPs, followed by chromatography to separate dNMPs. dUMP was dephosphorylated and analyzed as dUrd by HPLC (Fig. 3A) (see Materials and Methods). A similar procedure, with [^{3}H]FdUrd instead of [^{3}H]dUrd, was used to detect FdUMP in DNA (Fig. 3B). The results are summarized in Table 2.

dUMP was easily detected in DNA of FdUrd-treated cells. When the experiment was repeated with Ura present during the labeling period, the amount of dUMP in DNA increased 3- to 5-fold. This result can be accounted for by greater retention of dUMP in DNA due to the inhibitory effect of Ura upon Ura-DNA glycosylase (8–12). In previous experiments it was not possible to detect dUMP in DNA of untreated cells, indicating that there was less than 1 fmole of dUMP/μmole of DNA nucleotide (8).

FdUMP was also detected in DNA of FdUrd-treated cells at levels in the same order of magnitude as dUMP in DNA (Table 2). There was a pronounced increase (~12-fold) in FdUMP in DNA with increase of FdUrd in the culture medium from 0.1 μM to 1 μM, along with ~5-fold increase in intracellular FdUTP (Table 1). The amount of dUMP in DNA also increased 2- to 4-fold with increase in FdUrd in the medium, although, as noted above, there was no associated increase in intracellular dUTP (Tables 1 and 2) (see Discussion).

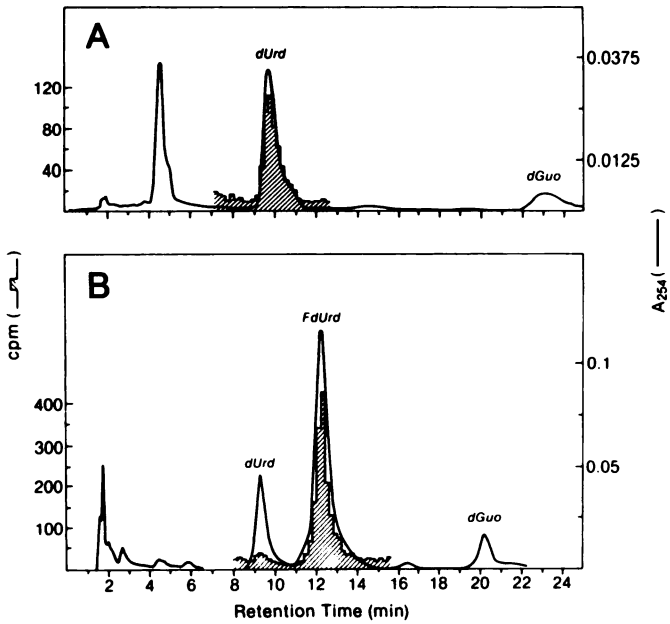

FIG. 3. *Analysis of dUMP and FdUMP in DNA by HPLC*

DNA isolated from cells treated with FdUrd (1 μM) and labeled with [^{3}H]dUrd (A) or [^{3}H]FdUrd (B) was digested to mononucleotides and chromatographed on paper (see Materials and Methods). dUMP or FdUMP was eluted, converted to deoxynucleoside, and analyzed by HPLC on reverse-phase columns, shown here. The A_{254} peaks of dUrd and FdUrd were from carriers; dGMP co-chromatographed with FdUMP and dUMP in the paper chromatography.

TABLE 2

dUMP and FdUMP in DNA

FdUrd in culture medium[a]	dUMP		FdUMP, −Ura
	−Ura	+Ura[b]	
μM	*pmoles/μmole DNA nucleotide*		
0.1	1.3[c]	5.9	0.4
1.0	4.6	13.8	4.9

[a] Total exposure to FdUrd was 6 hr, the last 2 hr of which included either [^{3}H]dUrd for measurements of dUMP in DNA or [^{3}H]FdUrd for measurements of FdUMP; total concentrations of FdUrd were 0.2 and 1.8 μM, respectively, during the 2-hr labeling period for measurements of FdUMP in DNA (see Materials and Methods).

[b] Ura included in the growth medium at 10 mM during the 2-hr labeling period.

[c] Average of two experiments.

DISCUSSION

The detection of dUTP in cells treated with methotrexate (4) indicated the possibility that FdUTP, which is degraded by dUTPase (2, 3), may be measured in cells treated with FdUrd; the results presented here show this to be the case. Intracellular FdUMP and FdUTP concentrations appear to correlate with levels of FdUrd in the growth medium. In contrast, intracellular concentrations of dUMP remained the same at the two levels of FdUrd that were tested. This is of interest because of the part that dTTP is thought to play in causing the increase in dUMP when activity of thymidylate synthetase is depressed (13).

dTTP inhibits three different enzyme reactions that lead to production of dUMP (13–15): reduction by ribonucleotide reductase of rUDP and rCDP to dUDP and dCDP, respectively; phosphorylation of dUrd to dUMP by thymidine kinase; and conversion of dCMP to dUMP by dCMP deaminase. At the higher level of FdUrd (1 μM) used here, intracellular dTTP was reduced to ~1/50 of its normal level [similar to the value found with methotrexate (4)], thus activating these three enzymes and helping to bring about the increase in dUMP, particularly as a result of the higher activity of dCMP deaminase (13, 16–19). Because of the fact that the elevation of dUMP at both levels of FdUrd was the same, even though intracellular dTTP was much lower with the higher FdUrd, it appears that the maximal level of dUMP is limited by factors not controlled by concentration of dTTP.

Incorporation of FUra into DNA was shown originally in a bacteriophage, the DNA of which normally contains Ura rather than Thy (20). However, the analogue was not found in DNA of normal cells treated with FUra (1). The explanation for this came from demonstration of the

FdUTPase activity of dUTPase (above) and the FUra-DNA glycosylase activity of Ura-DNA glycosylase (2, 3, 21). It has recently been shown that FUra or Ura may, in fact, appear in DNA of Thy-containing prokaryotes, particularly under circumstances of defective dUTPase, Ura-DNA glycosylase, and/or thymidylate synthetase (21–27).

It has not as yet been possible to detect Ura in DNA of animal cells under normal growth conditions (8), and this may be related in part to the much lower levels of dUTP in animal cells as compared with bacteria (4, 28). However, Ura has been detected in animal cells in which the activity of thymidylate synthetase was blocked by inhibition of dihydrofolate reductase with methotrexate (4), and this has been confirmed recently with a different inhibitor of dihydrofolate reductase (29).

The appearance of Ura in DNA of FdUrd-treated cells is not surprising considering the levels of dUTP found and the earlier results with dihydrofolate reductase inhibition. Given the comparable levels of FdUTP in the same cells and the fact that FdUTP also serves as substrate for DNA polymerases (30), it would be expected that significant incorporation of FdUMP into DNA takes place as well. The analyses of DNA provided direct evidence for this. The amounts of FUra in DNA correlated with FdUrd in media and intracellular levels of FdUTP; the amounts of Ura in DNA increased with increased FdUrd in media, although intracellular levels of dUTP remained the same at different drug concentrations. It is possible that with the higher amounts of FUra in DNA the capacity for removal of Ura and FUra (see below) was further exceeded, leading to additional retention of Ura as well.

The Ura and FUra found in DNA probably represent a very small fraction of the amounts actually incorporated, reflecting rapid turnover of Ura and FUra in DNA. In support of this is the 3- to 5-fold increase of Ura in DNA that occurs when removal of Ura from DNA by Ura-DNA glycosylase is inhibited by Ura (the addition of Ura had no effect on intracellular nucleotide pools). In addition, rapid "chase" of Ura from DNA was observed in earlier studies using methotrexate to inhibit thymidylate synthetase (8). The same effects are assumed to apply to FUra in DNA as well (2).

Ura-DNA glycosylase leaves an apyrimidinic site where Ura or FUra is removed, thus initiating a process of excision and repair (9). It is not clear at this time how much of the residual low level of DNA synthesis that persists in the inhibited cells is accounted for by abortive replication, and how much represents repair at sites of Ura/FUra removal or other lesions. In either case, the net result is active incorporation and removal of Ura, similar to the effect of methotrexate (8); however, with FdUrd as inhibitor, there is the additional participation of FUra in the repetitive insertion/removal process.

It is possible that fragmentation of DNA due to multiple sites of excision-repair results in irreversible changes in DNA that play a part in the cytotoxicity of FdUrd. Indirect evidence in support of this includes DNA fragmentation accompanying "thymineless death" in both prokaryotes and eukaryotes, and the apparent toxicity of elevated dUTP levels in prokaryotes (9, 22, 26, 27, 29, 31; see refs. 4 and 8 for additional review and references). Results of recent experiments show correlation of cytotoxicity, DNA fragmentation, and cellular levels of FdUrd or dUrd nucleotides, supporting the proposed mechanism for cytotoxicity.[3]

Note added in proof. While this paper was in press, a paper appeared by Kufe *et al.* (32) that also reports the identification of FdUMP in DNA of cells treated with FdURD.

[3] H. A. Ingraham, B. Y. Tseng, and M. Goulian, unpublished observations.

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