The Effect of Methotrexate on Levels of dUTP in Animal Cells*

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The amounts of intracellular nucleotides of dThd and dUrd were measured in cultured human lymphoblasts with and without treatment with methotrexate (plus hypoxanthine) to inhibit thymidylylate synthetase. dITP fell from ~40 pmol/10⁶ cells (untreated) to ~1 pmol/10⁶ cells with drug treatment. dUMP, which was at 0.8 pmol/10⁶ cells without the drug, increased ~10⁴-fold with methotrexate. dUTP was not detected in the cells without drug treatment (<0.3 pmol/10⁶ cells), but was easily measured at ~0.2 pmol/10⁶ cells in drug-inhibited cells. Thus, the ratio dUMP/dTTP increased from ~10⁻³ to ~10⁻⁵ in untreated cells to ~0.2 in cells treated with methotrexate, indicating that dUMP may be incorporated in substantial amounts into the DNA of the drug-treated cells. The magnitude of these effects on nucleotide pools suggests the possibility that the normal nucleolytic mechanism for removal of dUMP from DNA may play a part in the toxic effects on the cell that result from depression of thymidylylate synthetase activity.

Inhibition of thymidylate synthetase is known to result not only in depression of intracellular dTTP (28–32), but increase in dUMP, as well (29, 32–36). If the high intracellular concentrations of dUMP lead to significant increases in dUTP, this, together with the reduced dTTP, would be expected to favor greater incorporation of dUMP into DNA. Measurements of cellular dUTP pools have not been available previously, presumably because the levels are below limits of detection by methods ordinarily used.

In the studies described here, a sensitive procedure is used to determine dUTP levels in cells with and without inhibition of thymidylate synthetase with methotrexate. Combined with measurements of dTTP under the same conditions, the data allow predictions about relative rates of incorporation of dUMP and dTTP into DNA in normal and methotrexate-inhibited cells. The results suggest a possible mechanism for some of the toxic effects of such drugs that is related to cellular levels of dUTP and dTTP.

MATERIALS AND METHODS

Cell Culture and Labeling—Unless noted otherwise, the experiments were carried out on a cultured line of human lymphoblasts (8869), which were grown in suspension in modified Eagle’s medium as previously described (37). Hypoxanthine (25 μM) was added at the start of each experiment to avoid effects of methotrextate. Labeling with [3H]dUrd (0.1 μCi/mmol) was carried out during the last 2 h of drug treatment.

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with toluene and ethanol, the strips containing dUTP region then cut into 1-cm strips and counted in toluene scintillation fluid) in markers, the internal dUTP marker, and radioactivity (chromatogram markers, and overlap of internal markers with UV-absorbing components in some instances between internal and external markers). Recoveries of dUrd derived from the internal standard (dUTP) ranged between 10 and 40%, and this was used to correct the radioactivity in dUrd for losses during the procedure. Fractions with low radioactivity were counted a minimum of 60 min (6 x 10 min).

The procedure described above, carried out on untreated and methotrexate-treated lymphoblasts, was the source of most of the data presented in this report. Where noted, a modified procedure was used on untreated cells in an attempt to increase sensitivity for detection of dUTP. The modifications consisted of 1) oxidation of the extracts to periodate (40) just prior to the DEAE-Sephadex step; and 2) further purification, after dephosphorylation to dUrd, by paper chromatography in 1-butanol/NH₄OH (see below) before the final analysis by high pressure liquid chromatography. The procedure was otherwise as described above.

Specific Activity of [3H]dUrd-labeled Nucleotide Pools—Specific activity of [3H]dUMP in methotrexate-treated cells was determined on acid extracts of [3H]dUrd-labeled drug-treated cells (50-ml culture volumes) prepared as in the preceding, except that prior to acid extraction, the cells were washed once with 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, 10 mg/ml of bovine plasma albumin, and once with 0.02 M Tris/acetic acid, pH 6.8, 10 mg/ml of bovine plasma albumin, and the internal standard of unlabeled dUTP was omitted. After removal of trichloroacetic acid (see above), the extracts were chromatographed (22 h) directly on Schleicher & Schuell paper in ammonium isobutyrate (see above). dUMP, identified as the predominant peak of radioactivity (Fig. 1A), was eluted, dephosphorylated with phosphatase, and re-chromatographed on paper (Schleicher & Schuell 589 Orange Ribbon-C) in 1-butanol (water-saturated)/concentrated NH₄OH (100:1; v/v), or 1-butanol/water/formic acid (77:13:10; v/v/v), to purify dUrd (Fig. 1B). dUrd was analyzed for amount (Aₜ%), and radioactivity by high pressure liquid chromatography (see above) (Fig. 2). Specific activity of [3H]dTTP in untreated cells labeled with [3H]dUrd was determined using the same procedure as described for specific activity of [3H]UMP in drug-treated cells. The choice of dUMP and dTTP in untreated and treated cells, respectively, was based on their relative abundance in each case, to facilitate measurement of amount (Aₜ%) without interference from UV-absorbing contaminants. In several experiments specific activities of dUDP (methotrexate-treated cells), or dTDP and dTMP (untreated cells), were also determined and found to differ significantly from the specific activities of dUMP and dTTP, respectively.

Analysis of Intracellular dTTP in Methotrexate-treated Cells—Unlabeled lymphoblasts, treated with methotrexate (6 h) as described above, were extracted with trichloroacetic acid containing 1 pmol of [3H]dTPP (60 Ci/mmol)/10⁶ cells as internal standard. dTPP was purified by chromatography in ammonium isobutyrate, then dephosphorylated and the dThd was purified by chromatography in 1-butanol/NH₄OH (see above). Losses were corrected for by the radioactivity of the internal standard, and dThd was quantified by a sensitive enzymatic assay in which the dThd was phosphorylated to dTMP with thymidine kinase and [γ-32P]ATP. Samples containing 0.5 to 10 pmol of dThd were incubated 3 h in a mixture (20 µl) containing 50 mM Tris- HCl, pH 7.6, 5 mM MgCl₂, 0.5 mM MnCl₂, 5 µM [γ-32P]ATP (41) (1500 to 3000 cpm/pmol), 0.17 pmol of [3H]dThd (60 Ci/mmol), 1 mM dCDP (re-purified), 0.5 mg/ml of bovine plasma albumin, 10% glycerol, and Escherichia coli thymidine kinase (Fraction V) (42) sufficient to convert >95% dThd to dTMP. The mixture was chromatographed with dTMP and dThd markers on polyethyleneimine cellulose thin layer plates using 0.4 M LiCl. Paper chromatography in ammonium isobutyrate confirmed that the radioactive product was dTMP and not dUMP. 32P in dTMP gave the amount of dThd in the sample, easily detecting 0.2 pmol (Fig. 3). Conversion of the [3H]dThd to dTMP indicated completion of the reaction. Some
samples showed non-linearity in the kinase reaction before, but not after, the purification by paper chromatography in 1-butanol/NH₄OH.

Other Nucleotide Measurements—For untreated cells, the amounts of total acid-soluble radioactivity represented by dTTP, dTDP, and dTMP were determined on extracts from [³H]dUrd-labeled cells by paper chromatography in ammonium isobutyrate (Fig. 4A). These, together with the specific activity for dThd nucleotide (measured on dTTP, see above), gave the amounts of each dThd nucleotide. The dUMP and dUDP regions of the chromatograms (Fig. 4A) were eluted, dephosphorylated, and chromatographed (as dUrd) on paper in 1-butanol/NH₄OH to determine radioactivity in each; specific activity of dTTP was assumed to apply to dUrd nucleotides, as well, in untreated cells.

In [³H]dUrd-labeled methotrexate-treated cells, ~98% of the acid-soluble radioactivity was in dUDP (Fig. 4B). Approximately 2% of the radioactivity was in the region of dUDP on the chromatogram (Fig. 4B) and when this was analyzed as described above for dUDP in untreated cells it was found that about half was in dUDP. The remaining ~1% of radioactivity was present in another compound the identity of which is currently under investigation. The specific activity for [³H]dUMP in drug-treated cells (above) was used to convert radioactivities in dUMP and dUDP to picomoles, as described for the preceding.

Measurement of dUTP in Untreated NB Cells—At completion of labeling (see above), the medium was removed rapidly from the roller bottle and the cells (4.5 × 10⁸ determined as DNA) were covered immediately with 20 ml of cold trichloroacetic acid (10%) containing 50 nmol of dUDP. Total time from beginning removal of medium to complete immersion of cells in acid was ~15 s. The remainder of the procedure was the same as described above for lymphoblasts with the modifications to increase sensitivity for detection of dUTP, including periodate treatment and purification of the dUrd (derived from dUDP by paper chromatography before analysis by high pressure liquid chromatography (above). Specific activity of [³H]dUTP in the cell extract was determined as described for the lymphoblast cell line (above).

RESULTS

Measurement of Intracellular dUTP

Cells not Treated with Drug—In the procedure that was used to measure dUTP, deoxyuridylate pools were labeled by growing cells with [³H]dUrd. With the level of [³H]dUrd used here (0.1 μM), in 30 min, untreated cells incorporated 7 to 13% of total label from the culture medium into DNA, and another 2.0 to 2.2% was in the acid-soluble fraction, almost entirely in the form of deoxythymidine nucleotides (Fig. 4A). With a total intracellular deoxythymidine nucleotide pool of 50 to 90 pmol/10⁸ cells and generation time of 16 h, it is estimated that the turnover time for deoxythymidilate is ~6 min, and kinetic measurements show that there is ~80% of maximum uptake of [³H]dUrd into cellular pools by 5 min and maximal, and virtually constant, level from 20 to 60 min (data not shown). Thus, by 30 min, at which time cells were collected for extraction, the labeling conditions approached a steady state, and the specific activities of cellular nucleotides of dUrd and dThd were assumed to be very similar. Since the [³H]dUrd was diluted ~20-fold by cellular pools (see specific activity, below), the perturbation of cellular nucleotide pools by labeled nucleotides in the medium is assumed to be negligible at these concentrations, paralleling observations on the effects of similar concentrations of dThd upon cellular pools of deoxythymidilate (43). Using these labeling conditions, dUTP was isolated from the acid-soluble extract by successive paper chromatography (Fig. 5). A major function of the three paper chromatographic steps was to assure absence of dUDP, and to reduce contamination of the sample with the large amounts of radioactivity from dTTP, which migrates immediately ahead of dUTP (Fig. 5). The dUTP was converted to dUrd with phosphatase, and analyzed by high pressure liquid chromatography (Fig. 6). In spite of adequate recoveries of the dUTP internal standard (24 to 49%) (“Materials and Methods”), in three different experiments there was no radioactivity specifically associated with dUTP (Fig. 6). The identities of the radioactive components in the dUTP region of the paper chromatograms (Fig. 5 and 6) are not known.

An experiment was also carried out with additional steps to improve sensitivity for detection of small amounts of dUTP.
activity of dUTP, as well, the highest amount of radioactivity associated with dUTP (Fig. 7) corresponds to 0.3 fmol/10^6 cells. However, considering the small amounts (<1/3 background) and the remaining radioactive contaminants in close proximity (Fig. 7), the significance of this is uncertain.

**Methotrexate-treated Cells**—Cells were treated with methotrexate in the presence of exogenous sources of purine, serine,

![Figure 5](image5.png)

**Fig. 5.** Purification of dUTP-containing fraction from untreated cells. Acid-soluble extract from 200 ml of culture of [3H]-dUrd-labeled untreated cells, containing internal standard of unlabeled dUTP, was desalted on a column of DEAE-HCO_3^- and chromatographed on thick paper (Whatman 17) in ammonium isobutyrate (A) ("Materials and Methods"). Fractions from A containing dUTP (bracket) were eluted and chromatographed on an analytical grade paper in the same solvent system (B). The dUTP-containing fraction from B (bracket) was rechromatographed once again, as in B (C). The dUTP region from the third chromatogram (C) was pooled in two fractions (I, II).

By including oxidation with periodate (to remove ribonucleotides) and an additional purification step (as dUrd) the remaining UV components were eliminated, but small amounts of the closely associated radioactive components remained. In some fractions, a very small amount of radioactivity appeared in the position of dUrd in the chromatogram (Fig. 7). The specific activity for intracellular [3H]dTTP from cells labeled under the same conditions ranged from 537 to 648 cpm/pmol (three determinations), with an average of 590 ("Materials and Methods"). Using this value for the specific activity of dUTP, as well, the highest amount of radioactivity associated with dUTP (Fig. 7) corresponds to 0.3 fmol/10^6 cells. However, considering the small amounts (<1/3 background) and the remaining radioactive contaminants in close proximity (Fig. 7), the significance of this is uncertain.

![Figure 6](image6.png)

**Fig. 6.** Analysis by high pressure liquid chromatography of [3H]dUrd derived from [3H]dUTP in untreated cells. dUTP-containing fractions, I and II (Fig. 5C), were dephosphorylated with phosphatase and chromatographed on a reverse phase column. Effluent was analyzed for recovery of the internal standard (A260) and radioactivity in [3H]dUTP ("Materials and Methods"). A represents Fraction I, and B, Fraction II. Background (10 cpm) has been subtracted.

![Figure 7](image7.png)

**Fig. 7.** Analysis by high pressure liquid chromatography of [3H]dUrd derived from [3H]dUTP in untreated cells, using modified procedure. The procedure was carried out as described for Figs. 5 and 6 except that additional steps of periodate oxidation (prior to paper chromatography) and paper chromatography as [3H]dUrd (prior to high pressure liquid chromatography) were carried out to enhance sensitivity. One of the two final fractions is represented here.
and glycine to restrict the effects of the drug to inhibition of thymidylate synthetase. Here, the labeling period with [3H]-dUrd (2 h) was longer than for untreated cells, to assure equilibration with the expanded cellular pools of deoxyuridylate (29,32-36). Kinetic measurements of [3H]dUrd uptake into the acid-soluble fraction of drug-treated cells showed rapid initial uptake followed by slow linear rise for 4 h, with the value at 15 min 85% of the value at 2 h (not illustrated), indicating that labeling of deoxyuridylate pools approached steady state conditions at 2 h. Under these conditions, 0.015 to 0.02% of the total radioactive label in the medium was incorporated into DNA, and another 11 to 13% was present in the acid-soluble fraction from cells. Although there was a larger proportion of [3H]dUrd in the acid-soluble fraction (compared to untreated cells), dilution with the greatly expanded endogenous deoxyuridylate was 50-fold (see specific activity, below), again indicating an insignificant effect of the exogenous [3H]dUrd on cellular pool size.

The procedure for isolation of dUTP from the methotrexate-treated cells was the same as that described above for untreated cells. Here, the major radioactive component was dUMP (Fig. 4B), but this was removed with the first paper chromatogram, leaving dUDP as the major contaminant to be separated (Fig. 8, A and B), and this appeared to have been accomplished by the third paper chromatographic step (Fig. 8C).

In contrast to the results with untreated cells (above), samples from methotrexate-treated cells showed significant amounts of radioactivity associated with dUrd derived from dUTP (Fig. 9). The possibility that the radioactivity may have been from residual trailing contamination with dUDP (or an unidentified dUrd-containing compound located between dUDP and dUTP in isobutyrate chromatography) appears very unlikely, since: 1) the radioactivity in dUrd exceeds what was actually present in dUDP (Fig. 8C), even allowing for the lower efficiency for counting 3H-labeled nucleotide in paper (~one-third to one-sixth of the efficiency for Triton/toluene mixture); and 2) the ratio of radioactivity to A254 is essentially the same for the two fractions (Fig. 9, A and B), whereas it would be much larger for the fractions closer to dUDP (or the unknown compound) (Fig. 8, C, D) if there had been a significant contribution of radioactivity due to trailing.

The specific activity for [3H]dUMP in these cells ranged from 180 to 278 cpm/pmol (five experiments), with an average of 219. Using this for the specific activity of dUTP, and figures for recovery of purified dUTP from the internal standard (10 to 25%), the intracellular dUTP, for three different experiments, was 0.082, 0.222, and 0.264 pmol/10^6 cells.

### Cellular Levels of Other dUrd and dThd Nucleotides

Availability of the specific activity for [3H]dTTP in untreated cells facilitated estimation of cellular pools for the other dUrd and dThd phosphates (all of which were assumed to have the same specific activity as dTTP) by simple assessment of the proportion of total acid-soluble counts represented by each ("Materials and Methods"). The specific activity for dUMP in methotrexate-treated cells was used similarly to determine values for dUMP and dUDP. Because the specific activities of dThd and dUrd nucleotides were not the same in methotrexate-treated cells, dTTP was measured in these cells by an enzymatic assay for dThd using thymidine kinase and [γ-32P]ATP, after isolation as dTTP and conversion to dThd with phosphatase ("Materials and Methods").

The results are summarized in Table I. dUrd was -0.8 pmol/10^6 cells without drug treatment and this rose ~1400-fold with methotrexate. dUDP also increased greatly with drug treatment (~300-fold). The level for dTTP in untreated cells was ~40 pmol/10^6 cells, similar to previously reported values (32, 44, 45), and with the methotrexate treatment this fell to ~1 pmol/10^6 cells. As stated above, dUTP was not clearly detected in untreated cells (~0.3 fmol/10^6 cells), but with methotrexate treatment dUTP had risen at least 700-fold, to ~0.2 pmol/10^6 cells. Thus, with drug treatment the ratio of dUTP/dTTP had risen from ≤10^{-4} to ~0.2.

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**Fig. 8. Purification of dUTP-containing fractions from methotrexate-treated cells.** Acid-soluble extract from 200 ml of culture of [3H]dUrd-labeled methotrexate-treated cells was chromatographed on paper three successive times, selecting for the dUTP-containing fractions, as described for untreated cells (Fig. 5; "Materials and Methods"). (Background radioactivity has not been subtracted.)
**dUTP in Methotrexate-inhibited Cells**

Fig. 9. Analysis by high pressure liquid chromatography of \[^{3}H\]dUrd derived from \[^{3}H\]dUTP in methotrexate-treated cells. The dUTP-containing fractions, I and II (Fig. 8C), were converted to nucleoside and analyzed by recovery of internal standard (A~in~) and radioactivity in \[^{3}H\]U from \[^{3}H\]dUrd-labeled cells, as in Fig. 6. A represents Fraction I, and B, Fraction II.

**Table I**

Intracellular dThd and dUrd nucleotides

The procedures for the determinations are given under "Materials and Methods." Each represents the mean for three or more separate experiments except for dUDP, which was measured twice. Range is given in parentheses.

| Nucleotide | +Methotrexate | +Methotrexate |
|-----------|---------------|---------------|
| dTMP      | 7.4 (3.9-12.2) | ND            |
| dTDP      | 9.7 (7.1-12.6) | ND            |
| dTTP      | 39 (30-68)    | 0.98 (0.37-1.55) |
| dUMP      | 0.85 (0.41-1.3) | 1220 (920-1400) |
| dUDP      | 0.94 (0.63-0.063) | 15.6 (14.8-16.5) |
| dUTP      | \(\leq 3 \times 10^{-4}\) | 0.189 (0.082-0.264) |

* ND, not determined.

**dUTP in NB Cells**

To test whether the very low level of dUTP in untreated lymphoblasts is a peculiarity of that cell line, another cell line, SV-40-transformed human newborn kidney cells, was analyzed. Even with the modified procedure of greater sensitivity, no dUTP was detected in these cells (\(\leq 0.6 \text{ fmol}/10^6\) cells). The level of dTTP in the NB cells was 60 pmol/10^6 cells.

**DISCUSSION**

Inhibition of thymidylate synthetase in cultured lymphoid cells, resulting from inactivation of dihydrofolate reductase by methotrexate, caused a fall in dTTP to about one-fourth of its normal value. Reduction in dTTP pools with inhibition of thymidylate synthetase, measured by the DNA polymerase assay, has been reported several times previously, although in most cases the degree of reduction has not been as great as found here (28-32). The rise in dUMP accompanying the fall in dTTP has also been described before (29, 32-36); most of the earlier reports showed a much smaller effect on dUMP, e.g. up to a maximum of 13-fold increase (29, 32, 33, 35, 36), in contrast to the >10^3-fold increase found in the present study, although in one of the most recent studies, there was 300-fold increase in dUMP (34). It should be noted that the dUMP levels in untreated cells found in some of the earlier studies, which employed a thymidylate synthetase assay procedure (29, 33, 35, 36), were much higher (~10^2 to 10^3 times) than found here. Whether the differences are due to cell differences, type or amount of drug used, or the assay method, is not clear at this time. In more recent reports, the levels of dUMP determined both by the method used previously (12 to 15 pmol/10^6 cells) (32) and a modified form of the procedure (2 to 5 pmol/10^6 cells) (34) were closer to the values found in the current study.

Available evidence indicates that the increase in dUMP that occurs with inhibition of thymidylate synthetase is largely a result of "de-inhibition" of dCMP deaminase due to the fall in dTTP (32, 46-48). Additional contributions to the rise in dUMP come from the non-utilization by thymidylate synthetase, and increases in activity of ribonucleoside diphosphate reductase (converting UDP to dUDP, and CDP to dCDP) (32, 48-50), and thymidine kinase (32, 46, 51) (reducing excretion as dUrd), the enzyme activations in each case a result of the fall in dTTP.

The high dUMP level is not at present known to have physiological consequences, although one effect may be to exert some control upon its own level. It has been shown that dUMP inhibits the activity of dCMP deaminase \((K_i = 0.21 \text{ mm})\) (32). With the dUMP concentrations reported here in drug-treated cells, inhibition of dCMP deaminase may play a part in preventing even further increase in dUMP, although this mechanism may be of little significance for lower levels of dUMP (29, 32, 33, 35, 36). An additional effect of the high levels of dUMP may be to limit rate of utilization of exogenous thymidine by competing at the level of dTTP kinase. This may account, in part, for the poor incorporation of \[^{3}H\]dThd in methotrexate-treated cells. It has also been suggested that reduced incorporation of \[^{3}H\]dThd into DNA under similar circumstances may result from competition of dTThd with increased dUrd at the thymidine kinase step (32), but, in contrast to a previous report (32), there was little or no evidence for intracellular accumulation of dUrd in the experiments reported here.

It is shown in the present study that the increase in dUMP in methotrexate-treated cells is associated with a marked increase in dUTP, as well, presumably via dTTP kinase and nucleoside diphosphate kinase. It was not known before these results whether significant accumulation of dUTP would ensue in the face of the highly effective mechanism opposing it. The enzyme dUTPase, which hydrolyzes dUTP to dUMP and PP\(_i\), functions as a major factor in preventing accumulation of dUTP (2-7, 16, 17, 20, 21, 25-26). Intracellular concentrations of dUTP normally are extremely low primarily because of this mechanism and the present analyses give a figure, not previously available, for an upper limit in untreated cells. The figure may be compared with indirect estimates of ~0.5 \(\mu\)M for \(E. coli\) (7) and 1/200 of dTTP concentration (18). Using the estimate of 1 \(\mu\)l for intracellular volume of 10^6 cells, the dUTP concentration in lymphoid cells is ~0.3 nM. It is possible that a higher level of dUTP in \(E. coli\) compared to the level in animal cells reflects the utilization by prokaryotes of the dUTPase reaction as the major source of dUMP (52, 53). In prokaryotes, dCTP is converted to dUTP by dCTP deaminase, which is absent in animal cells.

Although not detected in untreated cells, dUTP was easily

\(\text{(1)}\) M. Goulain, B. Bleile, and B. Y. Tseng, unpublished results.
measured in methotrexate-treated cells at ~0.2 pmol/10^6 cells. The concentration of dUTP in untreated cells is far below the K_m for dUTPase (variably found to be between 0.1 and 3 μM (5, 6, 24, 54)). With methotrexate treatment, dUTP evidently rises until it reaches a level at which the rate of hydrolysis by dUTPase equals the greatly increased rate of formation of dUTP under these circumstances.

Since DNA polymerases utilize dUTP (8, 9), the approach of dUTP concentration to that of dTTP in the methotrexate-treated cell implies that significant incorporation of dUMP into DNA must occur in the course of the residual DNA synthesis in these cells. The presence of dUMP in DNA activates a mechanism for its removal which is initiated by removal of the base Ura by the enzyme, Ura-DNA glycosylase (7, 12–15). The resulting apurinic/apyrimidinic site is incised by an apurinic/apyrimidinic endonuclease, followed by excision and repair (13, 20, 55, 56).

It is commonly assumed that the principal mechanism for cytotoxicity in methotrexate-treated cells is a direct result of levels of dTTP being inadequate to sustain synthesis. To what extent a fall in dTTP of the magnitude observed here, by itself, affect the rate of DNA synthesis, with or without causing cell death, is not at present known.

The current studies point to a process that results both directly and indirectly from reduced dTTP concentration, and which may also have toxic consequences for the cell. In the course of gap filling for excision/repair at the site of dUMP insertion (see above), there is a significant probability of reinsertion of dUMP, resulting in reinitiation of the process of Ura removal/incision/excision/repair. This probability is a function of repair patch size and ratio of concentrations, dUTP/dTTP. As the concentrations of dUTP and dTTP approach each other, a point may be reached at which the rate of dUMP incorporation into DNA exceeds the net rate of dUMP removal, thus leading to cyclic repetition of the insertion/removal process, without gap closure and possibly even progressive net gap widening. Even if this point is not reached, the active process of incorporation/removal may result in double strand interruptions (especially if both strands are involved) or other irreversible alteration(s) in DNA structure.

In experiments reported elsewhere (57), it is shown that dUMP is present in DNA of cells treated with methotrexate, thus fulfilling the prediction that follows from the measurements of dUTP and dTTP pools presented here. In addition, the dUMP in DNA increases with inhibition of Ura-DNA glycosylase (with Ura), and disappears rapidly if dThd is added to the culture, reflecting the active process of removal that accompanies dUMP incorporation (57).

In vitro experiments illustrate the fragmentation of DNA that may result from dUMP incorporation/removal at ratios of dUTP to dTTP of 1:10 or greater (24–27). In one of the in vitro systems the fragmentation is irreversible, i.e. maturation to high molecular weight DNA does not ensue with removal of dUTP (24, 27). Although some dut or sos (dUTPase-defective) mutants of E. coli are able to grow essentially normally in spite of the greater fragmentation of newly synthesized DNA that results from the (presumed) higher levels of dUTP (16, 17, 58), strains with the most severe defect in dUTPase do show a growth defect (58).

Fragmentation of DNA has been observed previously in association with "thymine-less" states, both in thymine-requiring prokaryote mutants (59-64) and drug-treated animal cells (65-67). The possibility of a relationship of fragmentation to cytotoxicity is supported by the observation (in a prokaryote) that both are reduced by a defect in Ura-DNA glycosylase (22). However, additional information will be needed to establish what, if any, role is played by dUMP incorporation/removal in the cytotoxicity that results from inhibition of thymidylate synthetase.

REFERENCES

1. Friedkin, M. (1973) Adv. Enzymol. 38, 235–292
2. Bertani, E., Haggmark, A., and Reichard, P. (1963) J. Biol. Chem. 238, 67–68
3. Bertani, E., Haggmark, A., and Reichard, P. (1963) J. Biol. Chem. 238, 3407–3413
4. Greiberg, G., and Somerville, R. (1963) Proc. Natl. Acad. Sci. U.S.A. 48, 247–257
5. Williams, M. V., and Cheng, Y-C. (1979) J. Biol. Chem. 254, 2897–2901
6. Ingraham, H. A., Tseng, B. Y., and Goulain, M. (1980) Cancer Res. 40, 998–1001
7. Shibata, J., and Kornberg, A. (1978) J. Biol. Chem. 253, 3305–3312
8. Richardson, C. C., Schildkraut, C. L., and Kornberg, A. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 9–19
9. Dube, D. K., Kunkel, T. A., Seal, G., and Loeb, L. A. (1979) Biochim. Biophys. Acta 581, 369–382
10. Hanawalt, P. C., Cooper, P. K., Jameson, A. K., and Smith, C. A. (1979) Annu. Rev. Biochem. 48, 783–836
11. Talpaert-Borle, M., Clerici, L., and Campagnari, F. (1979) J. Biol. Chem. 254, 6387–6391
12. Lindahl, T., Loungvist, S., Siegert, W., Nyberg, B., and Sperans, B. (1977) J. Biol. Chem. 252, 3286–3294
13. Lindahl, T. (1979) Prog. Nucleic Acids Res. Mol. Biol. 22, 135–192
14. Sekiguchi, M., Hayakawa, H., Makino, F., Tanaka, K., and Okada, Y. (1976) Biochim. Biophys. Acta Commun. 33, 293–298
15. Caradonna, S. J., and Cheng, Y-C. (1980) J. Biol. Chem. 255, 2293–2300
16. Tye, B-K., Nyman, P-O., Lehman, I. R., Hochhouser, S., and Weiss, B. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 154–157
17. Tye, B-K. and Lehman, I. R. (1977) J. Mol. Biol. 117, 293–306
18. Olivera, B. M., Manlapaz-Ramos, P., Warner, H. R., and Duncan, R. B. (1979) J. Mol. Biol. 126, 265–275
19. Warner, H. R., and Duncan, B. K. (1977) DNA Synthesis, Present and Future, pp. 267–279, Plenum Press, New York
20. Weiss, B., Rogers, S. G., and Taylor, A. F. (1978) in DNA Repair Mechanisms (Hanawalt, P. C., Friedberg, E. C., and Fix, C. F., eds) pp. 191–194, Academic Press, New York
21. Tye, B-K., Chien, J., Lehman, I. R., Duncan, B. K., and Warner, H. R. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 233–237
22. Makino, F., and Munakata, N. (1978) J. Bacteriol. 134, 24–29
23. Tamanoi, F., and Okazaki, T. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2195–2199
24. Brynolf, K., Eliasson, R., and Reichard, P. (1978) Cell 13, 573–580
25. Graffstrom, R., Tseng, B., and Goulain, M. (1978) Cell 15, 131–140
26. Wix, E., Uhlem, O., and Krokan, H. (1978) Biochim. Biophys. Acta 520, 253–270
27. Tseng, B. Y., Graffstrom, R. H., Revie, D., Oertel, W., and Goulain, M. Cold Spring Harbor Symp. Quant. Biol. 43, 283–290
28. Tattersall, M. H. N., and Harrap, K. R. (1973) Cancer Res. 33, 3086–3090
29. Tattersall, M. H. N., Jackson, R. C., Conners, T. A., and Harrap, K. R. (1973) Eur. J. Biochem. 9, 733–739
30. Fridland, A. (1973) Cancer Res. 34, 1883–1888
31. Skoog, L., Nordenskjöld, B., Humla, S., and Hägerström, T. (1976) Eur. J. Cancer 12, 163–171
32. Jackson, R. C. (1978) J. Biol. Chem. 253, 7440–7446
33. Myers, C. E., Young, R. C., and Chabner, B. A. (1975) J. Clin. Invest. 56, 1231
34. Moran, R. G., Spears, C. P., and Heidelberger, C. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1456–1460
35. Klubes, P., Connelly, K., Cerna, I., and Mandel, H. G. (1978) Cancer Res. 38, 2325–2331
36. Ardalan, B., Buscaglia, M. D., and Schein, P. S. (1978) Biochem. Pharmacol. 27, 2009–2103
37. Tseng, B. Y., and Goulain, M. (1975) J. Mol. Biol. 99, 317–337
38. Shein, H. M., and Enders, J. F. (1962) Proc. Soc. Exp. Biol. Med. 109, 495–500
39. Krstulovic, A. M., Brown, P. R., and Rosie, D. M. (1977) Anal. Chem. 49, 2237–2241
40. Garrett, C., and Santi, D. V. (1979) Anal. Biochem. 99, 268–273
dUTP in Methotrexate-inhibited Cells

41. Glynn, I. M., and Chappell, J. B. (1964) Biochem. J. 90, 147-149
42. Okazaki, R., and Kornberg, A. (1964) J. Biol. Chem. 239, 269-274
43. Bjursell, G., and Reichard, P. (1973) J. Biol. Chem. 248, 3904-3909
44. Skoog, K. L., Nordenskjöld, B. A., and Bjursell, K. G. (1973) Eur. J. Biochem. 33, 428-432
45. Bjursell, K. G., Reichard, P., and Skoog, K. L. (1972) Eur. J. Biochem. 33, 428-432
46. Maley, F., and Maley, G. (1962) Biochemistry 1, 847-851
47. Scarano, E., Geraci, G., Polzella, A., and Campanile, E. (1963) J. Biol. Chem. 238, PC1556-1557
48. Robert de Saint Vincent, B., Dechamps, M., and Buttin, G. (1980) J. Biol. Chem. 255, 162-167
49. Moore, E. C., and Hurlbert, R. B. (1966) J. Biol. Chem. 241, 4902-4909
50. Thelander, L., and Reichard, P. (1979) Annu. Rev. Biochem. 48, 133-158
51. Ives, D. H., Morse, P. A., and Potter, V. R. (1962) Fed. Proc. 21, 383
52. O'Donovan, G. A., Edlin, G., Fuchs, J. A., Neuhard, J., and Thomassen, E. (1971) J. Bacteriol. 105, 666-672
53. Neuhard, J., and Thomassen, E. (1976) J. Bacteriol. 126, 999-1001
54. Wist, E. (1979) Biochim. Biophys. Acta 565, 98-106
55. Lian, S. (1978) in DNA Repair Mechanisms (Hanawalt, P. C., Friedberg, E. C., and Fox, C. F., eds) pp. 175-178, Academic Press, New York
56. Friedberg, E. C., Bonura, T., Gone, R., Simmonds, R., and Anderson, C. (1978) in DNA Repair Mechanisms (Hanawalt, P. C., Friedberg, E. C., and Fox, C. F., eds) pp. 163-173, Academic Press, New York
57. Goulian M., Bleile, B., and Tseng, B. Y. (1980) Proc. Natl. Acad. Sci U. S. A., 77, 1956-1960
58. Hochhauser, S. J., and Weiss, B. (1978) J. Bacteriol. 134, 157-166
59. Freifelder, D. (1969) J. Mol. Biol. 45, 1-7
60. Freifelder, D., and Katz, G. (1971) J. Mol. Biol. 57, 351-354
61. Nakayama, H., and Hanawalt, P. (1975) J. Bacteriol. 121, 537-547
62. Yoshinaga, K. (1976) Biochim. Biophys. Acta 294, 204-213
63. Walker, J. R. (1970) J. Bacteriol. 134, 1391
64. Mennigmann, H-D., and Szybalski, W. (1962) Biochem. Biophys. Res. Commun. 9, 398
65. Salzman, N. P., and Thorén, M. M. (1973) J. Virol. 11, 721-729
66. Perlman, D., and Huberman, J. A. (1977) Cell 12, 1028-1043
67. Laipis, P. J., and Levine, A. J. (1979) Virology 88, 588-593