Antifolate-induced misincorporation of deoxyuridine monophosphate into DNA: Inhibition of high molecular weight DNA synthesis in human lymphoblastoid cells

(metoprine/DNA synthesis/lipid-soluble antifolate)

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ABSTRACT   *In vitro* exposure of a human lymphoblastoid cell line (WIL-2) to the antifolate metoprine (DDMP), when followed by the addition of exogenous deoxyuridine, led to intracellular accumulation of deoxyuridine triphosphate (dUTP) and incorporation of deoxyuridine monophosphate (dUMP) into DNA. When newly synthesized DNA was extracted from DDMP-treated cells that had been labeled with deoxyuridine for up to 3 min, most of the DNA synthesized was no larger than 4 S on alkaline sucrose gradients. In contrast, the predominant form of newly synthesized alkali-stable DNA in cells not treated with drug was larger than 4 S. Abnormal progression of DNA synthesis, degradation of newly synthesized DNA, or both occurred as a delayed consequence of DDMP treatment in the absence of exogenous deoxyuridine when thymidine was used to label DNA of DDMP-treated WIL-2 cells. Although the toxic impact on cell viability and genetic stability of antifolate-induced misincorporation of dUMP into DNA was not elucidated, it was clear that antifolates can directly perturb the quality as well as the quantity of DNA synthesized by drug-treated cells.

Prokaryotic and eukaryotic cells contain a potent deoxyuridine triphosphatase (1–4) and a uracil *N*-glycosylase-dependent repair system (5–9) that normally prevent accumulation of dUMP-containing DNA. We have found that, when intact WIL-2 human lymphoblastoid cells are inhibited by antifolates methotrexate (MTX) or metoprine [2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine, DDMP], the apparent levels of intracellular dUMP can be proportionally increased by exposure to increasing exogenous concentrations of deoxyuridine (10). In this study we examined the fate of exogenously added deoxyuridine in DDMP-treated cells, in order to determine whether dUTP becomes detectable and leads to dUMP misincorporation. We also looked for perturbations of DNA synthesis that might occur as a result of this lesion. Specifically, it was important to determine whether antifolates and deoxyuridine might affect the normal progression of DNA synthesis and not simply decrease overall incorporation into DNA products of all sizes.

MATERIALS AND METHODS

Chemicals and Reagents. [6-³H]Deoxyuridine (24.2 Ci/mmol), [*methyl*-³H]thymidine (49.5 Ci/mmol), [*methyl*-³H]deoxythymidine 5′-triphosphate, tetrasodium salt (63.2 Ci/mmol), and [5-³H]deoxyuridine 5′-triphosphate (13.5 Ci/mmol) were obtained from New England Nuclear and Amersham/Searle (1 Ci = 3.7 × 10¹⁰ becquerels). Unlabeled nucleotide precursors were purchased from P-L Biochemicals, cellulose thin-layer chromatography sheets from Eastman, polyethylene-imine (PEI)-cellulose-F sheets from MCB Chemical (Norwood, OH), and DEAE-cellulose paper from Whatman.

Snake venom phosphodiesterase and deoxyribonuclease were obtained from Worthington, and bacterial alkaline phosphatase from Sigma. MTX was provided by the Developmental Therapeutics Program, Division of Cancer Treatment of the National Cancer Institute, and DDMP was a gift from Carl Sigel and C. A. Nichol (Burroughs Wellcome, Research Triangle Park, NC).

Cell Culture Maintenance. The human lymphoblastoid cell line, WIL-2, utilized in this study was obtained and maintained as described (11). Prior to each experiment the cells were incubated for 1 hr in fresh minimal essential medium supplemented with 10% fetal calf serum [dialyzed against three changes of 100 vol of 0.15 M NaCl for a period of 48 hr or as obtained directly from Baltimore Biological Laboratory (Cockeysville, MD)]. The cell suspensions were then centrifuged at 500 × *g* for 5 min and resuspended in fresh minimal essential medium.

Sample Preparation for Nucleotide Pool Analysis. Cells were incubated in fresh growth medium for 15 min prior to addition of [³H]deoxyuridine. After addition of [³H]deoxyuridine, cells were harvested at the indicated times by centrifugation for 30 sec in an Eppendorf microcentrifuge at 12,800 × *g*, which immediately removed the cells from the labeling medium. The supernatant was then discarded and the pellet was suspended in 60% (vol/vol) methanol.

For rapid pulse experiments, cells were injected directly into 60% methanol at −20°C. Extraction of nucleosides and nucleotides was carried out for at least 24 hr before further processing of the samples.

Nucleotide Pool Analysis by Cellulose Thin-Layer Chromatography. The methanol nucleotide pool extracts described above were lyophilized to dryness and resuspended in 30 μl of 10% isopropyl alcohol/10% acetic acid/80% water (vol/vol). DNA nucleotides (0.01 M) were used as standards for chromatography. Cellulose plastic plates (Kodak), impregnated with fluorescent dye indicator, were divided into 10 numbered channels. Portions of each sample were spotted at the origin, positioned at 2 cm from the bottom of the plate for each channel. Portions of standard solutions were spotted together with or beside each sample, and the position of migration was visualized by irradiating the plates with shortwave ultraviolet light. The solvent system utilized for the nucleotide separations was isobutyric acid/NH₄OH/H₂O (63:1:33 vol/vol). Thin-layer chromatography plates were developed for approximately 4 hr (or until the solvent had moved 17 cm from the bottom of the plate)

Abbreviations: MTX, methotrexate; DDMP, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (metoprine); PEI, polyethyleneimine.

and then air dried overnight. The dried sheets were cut into strips following the demarcation of each channel, and the strips were cut further into 0.5-cm sections and placed in scintillation vials for measurement of radioactivity. The position of the radioactive nucleotides was then recorded relative to the reference markers in the same or adjacent channels.

PEI-Cellulose Chromatography of Nucleotides. PEI thin-layer chromatography was carried out essentially as described (12). Dye-impregnated PEI-cellulose plastic plates, prewashed in 10% NaCl solution up to 5 cm from the bottom edge of the plate, were transferred without drying to distilled water and washed two times to the top edge of the sheet. Plates were then air dried overnight and stored in the dark at 4°C until needed. The washed plates were divided into channels, as described above, and samples were applied for chromatography. Plates were developed in 1 M acetic acid, which was allowed to migrate to 4 cm from the origin. The chromatography sheet was then transferred, without drying, to a chromatography tank containing 0.3 M LiCl, and the elution solution was allowed to migrate to approximately 10 cm from the sample origin. The sheets were then air dried at room temperature and radioactivity was determined as described above (for cellulose chromatography plates). This method resolved the nucleoside monophosphates (dNMP), diphosphates (dNDP), and triphosphates (dNTP).

Procedure for Digestion of Nucleotides to Nucleosides. Nucleotide samples as isolated from chromatography plates were flash evaporated in a Buchler rotary evaporator at 40°C and resuspended in a 0.11 M Tris·HCl buffer, pH 8.9, containing 0.1 M NaCl and 15 μM MgCl. Snake venom phosphodiesterase solution (0.4 unit per sample) was added along with bacterial alkaline phosphatase (0.1 unit per sample) and the nucleotide and enzyme mixture (250 μl) was incubated for 3 hr at 37°C, a period of time that ensured complete digestion of nucleotides to nucleosides. The digested samples were concentrated by lyophilization and applied to cellulose chromatography sheets, prepared as described above. The sheets were developed in butanol/4% boric acid solvent (86:14, vol/vol), to separate the nucleosides. The chromatography plates were dried, cut, and assayed as described above. RNA nucleotides migrated together to only a short distance from the origin, allowing resolution of the DNA nucleosides, deoxyuridine, thymidine, and deoxycytidine.

Extraction of DNA and Digestion of DNA to Nucleosides. DNA from the pellets extracted with 60% methanol was isolated by NaDodSO$_4$/phenol extraction, following the procedure described by Kidson (13). The extracted DNA was resolubilized by means of overnight dialysis against 0.15 M NaCl containing 0.25 μM MgCl. DNA samples were digested with 60 units of DNase I per sample for 2 hr at 37°C. After digestion with DNase I an equal volume of Tris·HCl buffer, pH 8.9, containing 0.1 M NaCl, and an enzyme solution consisting of 60 units of snake venom phosphodiesterase and bacterial alkaline phosphatase were used to digest the preparation to nucleosides as described above. The resulting digests were concentrated by lyophilization and nucleosides were separated by thin-layer chromatography.

Alkaline Sucrose Centrifugation of DNA. The procedure used for alkaline sucrose centrifugation of DNA was modified from Perlman and Huberman (14). Extracts were prepared by directly injecting the labeled samples into 60% methanol at −20°C, followed by incubation at this temperature for at least 24 hr before further processing. This procedure allows analysis of both soluble pool and DNA to be carried out on the same samples. Cell pellets were prepared for gradient analysis by resuspending them in 0.3 M NaOH/2% NaDodSO$_4$ solution and extracting then for 3 hr at room temperature on a Fisher

(Roto Rack) rotator at 4 rpm. Alkaline sucrose centrifugation was performed in Beckman SW41 polypropylene centrifuge tubes on gradients of 10–30% (wt/vol), alkaline sucrose containing 0.7 M NaCl, 0.3 M NaOH, 10 mM Tris, and 1 mM EDTA. Alkaline extracts of the cell pellets were poured onto the gradients and centrifuged for 18 hr at 38,000 rpm at 14°C. The gradients were fractionated into 0.3-ml aliquots. One hundred microliters of each fraction was pipetted onto Whatman 3 MM paper discs, which were processed for measurement of radioactivity as previously described. Samples were also analyzed for incorporated radioactivity by adsorbing aliquots of the fractions on DEAE chromatography paper (12). Bovine serum albumin, which sediments identically in alkaline and neutral sucrose gradients, was used as an internal centrifugation standard.

RESULTS

Effect of DDMP on dUMP, dUDP, and dUTP Pools. Inhibition of cellular DNA synthesis by DDMP led to apparent accumulation of intracellular pools of dUMP; this could be greatly increased by addition of deoxyuridine to the medium
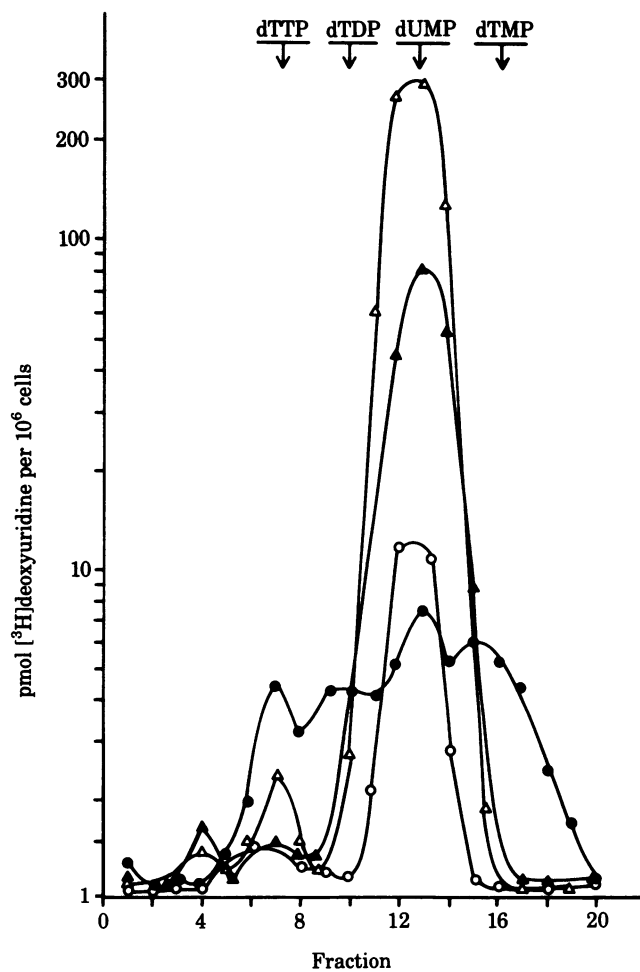

FIG. 1. Deoxyuridine incorporation into the soluble pools of WIL-2 cells. DDMP (5 μM) was added to cultures and incorporation of the indicated concentrations of deoxyuridine was determined after collection of WIL-2 cells by centrifugation. Fifteen minutes after addition of 5 μM DDMP, [³H]deoxyuridine, or both to WIL-2 cells (1 × 10⁷ per ml), the soluble pools of nucleotide were extracted with 60% methanol at −20°C. Samples were subsequently chromatographed on cellulose thin layers, using the isobutyric acid solvent system (isobutyric acid/NH$_4$OH/H$_2$O; 66:1:33). ●, 2.5 μCi of 0.1 μM deoxyuridine; ○, 2.5 μCi of 0.1 μM deoxyuridine, 5 μM DDMP; ▲, 2.5 μCi of 1.0 μM deoxyuridine, 5 μM DDMP; △, 2.5 μCi of 5.0 μM deoxyuridine, 5 μM DDMP.

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(Fig. 1). Further experiments showed that dUTP and dUDP could also be detected as potential DNA precursors in cells incubated with 5 μM DDMP and deoxyuridine. Identification of deoxyuridine as the major nucleoside constituent of dNMP, dNDP, and dNTP isolated from DDMP-treated cells was accomplished by methanol extraction, separation of the nucleotides by thin-layer chromatography, and enzymatic digestion of the eluted nucleotides to nucleosides, followed by rechro-

matography as shown in Fig. 2. The nucleotide composition of these same components was then confirmed by PEI-cellulose thin-layer chromatography (Fig. 3). Because no attempt was made to quantitate adventitious breakdown of dUTP, the significance of the relatively high levels of dUDP observed in these experiments is not clear.

dUMP Incorporation into DNA. DNA synthesized in the presence of DDMP and [^{3}H]deoxyuridine was analyzed for nucleotide incorporation by injection of samples directly into methanol at $-20°C$, followed by processing as described above. After 90-sec periods of labeling, most of the [^{3}H]deoxyuridine label in DNA was present as dUMP (Fig. 4). The butanol/boric
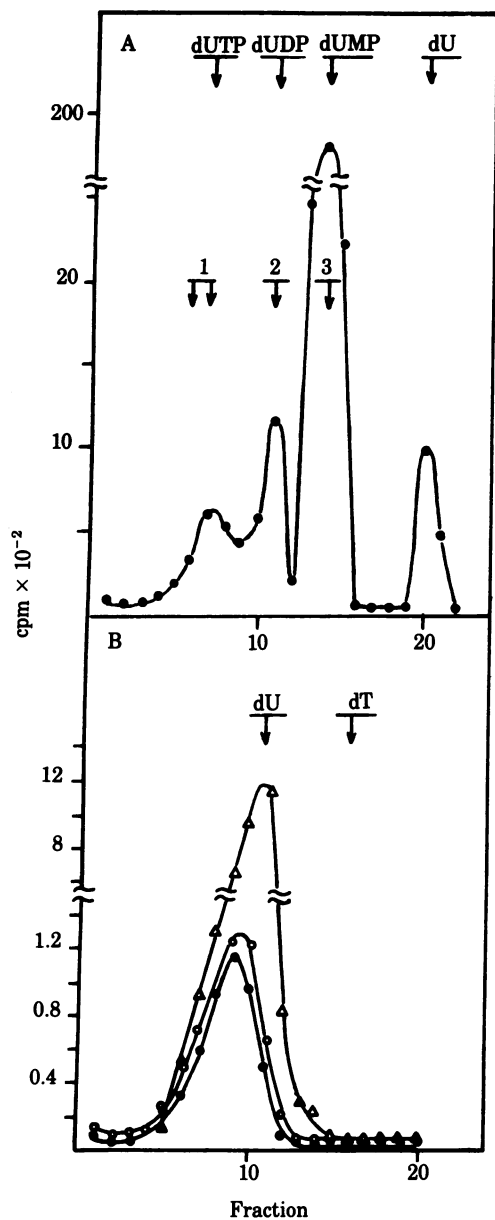

FIG. 2. Base composition of deoxyuridine products in DDMP-treated cells. DDMP (5 μM) was incubated with to 2×10^7 WIL-2 cells for 15 min together with [^{3}H]deoxyuridine (100 μCi/ml; 4.13 nmol). Samples were harvested by injection into 60% methanol. (*A*) Nucleotides that were separated by isobutyric acid/NH$_4$OH/H$_2$O thin-layer chromatography as described in Fig. 1. The nucleotides chromatographing to positions 1, 2, and 3 were then eluted in isopropyl alcohol from the thin-layer chromatography plate and hydrolyzed enzymatically to nucleosides. These samples were subsequently applied to another cellulose thin-layer plate and chromatographed for separation of deoxyuridine (dU) from thymidine (dT), and from ribonucleosides, with the butanol/boric acid solvent system. The samples in *B* are designated as eluted from position 1 (●), position 2 (○), and position 3 (△) of the chromatogram shown in *A*.
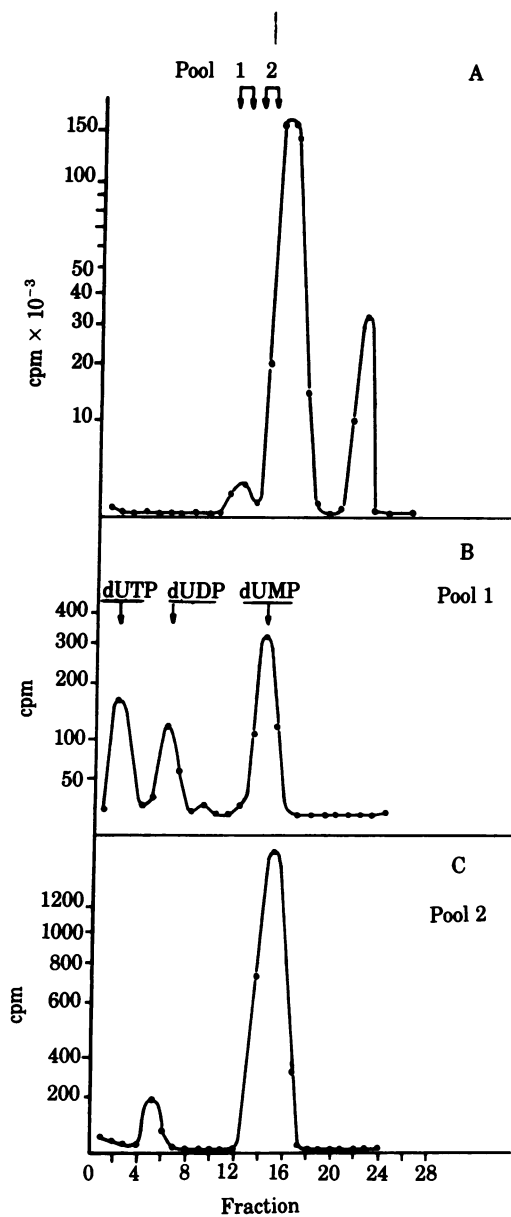
FIG. 3. Identification of nucleotide products of deoxyuridine isolated from DDMP-treated cells. DDMP (5 μM) was added to 2×10^7 WIL-2 cells with 100 μCi of [^{3}H]deoxyuridine (4.13 nmol). After 15 min of incubation the cells were harvested by centrifugation and the cell pellet was extracted with 60% methanol at $-20°C$. The isolated nucleotides and nucleosides were then chromatographed on cellulose thin-layer plates, using the isobutyric acid/NH$_4$OH/H$_2$O solvent system (*A*). The fractions indicated by the arrow and brackets were subsequently pooled and the nucleotides were eluted in isopropyl alcohol. These samples were then rechromatographed on PEI-cellulose plates to characterize the nucleotide composition of the fractions (*B* and *C*).
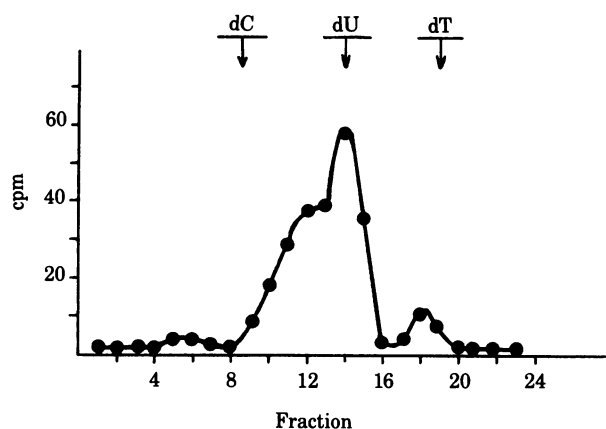
FIG. 4. Incorporation of dUMP into newly synthesized DNA in cells exposed to DDMP and deoxyuridine. Cells were treated as described in Fig. 3. After harvesting of the cells, the pellet from the methanol extraction was further extracted by phenol and NaDodSO₄, and DNA was then digested and analyzed for nucleotide composition.

acid thin-layer chromatography procedure separates deoxyuridine, deoxycytidine, and thymidine from each other and from ribonucleotides. Unlabeled nucleosides were included as markers with the chromatography samples as well as in channels on each side of the samples to confirm the identity of each nucleoside.

Effect of DDMP on DNA Synthesis. The progression of DNA synthesis at the replication fork was examined in an at-

tempt to detect perturbation of normal DNA synthesis (Fig. 5 A, B, and C). Under drug-free conditions, [³H]thymidine was incorporated as expected into 4 S "Okazaki-sized" fragments during short labeling periods. High molecular weight DNA became predominantly labeled with longer pulses of 120 and 300 sec.

Fig. 5 D, E, and F shows the gradient analysis of samples pulse labeled with thymidine for 30, 120, and 300 sec after 15 min of exposure to 5 μM DDMP. Synthesis of high molecular weight DNA progressed similarly to that in control cells during pulse labeling intervals of short duration when thymidine was added as an exogenous precursor (Fig. 5 D and E), an apparent result of thymidine's ability to rescue cells from their deficiency of thymidine nucleotide. In contrast to the situation in untreated cells, our results indicated that abnormal progression of DNA synthesis, or degradation of newly synthesized high molecular weight DNA, occurred as a delayed consequence of the antifolate-induced lesion in cells labeled for long pulse intervals (5 min) (Fig. 5F).

Synthesis of DNA in DDMP-treated cells that had been incubated with thymidine as a labeled exogenous precursor contrasted with that seen when only deoxyuridine was added as a labeled DNA substrate. Under these conditions, overall incorporation of deoxyuridine was markedly inhibited, and progression of synthesis to DNA larger than "Okazaki fragments" was blocked (Fig. 6 D, E and F). In the absence of DDMP, on the other hand, deoxyuridine was incorporated as dTMP (Fig. 6 A–C). In this circumstance 4S DNA was synthesized at all pulse labeling times, but larger DNA became the predominantly labeled product as duration of pulse increased. The ratio of deoxyuridine incorporation into DNA of 4.3 S and smaller (fractions 24–33 in each gradient shown in Fig. 6), in control vs. DDMP-treated samples was 39:1 at 40 sec, 33:1 at 120 sec and 16:1 at 180 sec. On the other hand, the ratio of labeling of large DNA (fractions 1–23) was 45:1 at 40 sec, 71:1 at 80 sec, and 73:1 at 180 sec, indicating a preferential block to synthesis of large
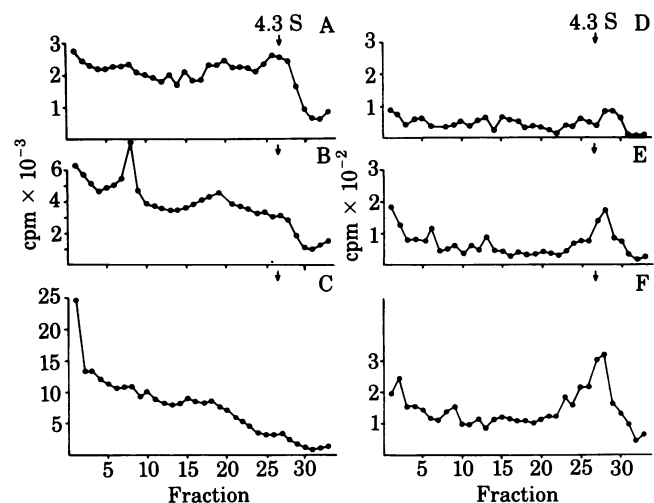
FIG. 6. Incorporation of deoxyuridine into small DNA fragments in the presence of DDMP. Untreated (*A–C*), and DDMP-pretreated (*D–F*) (5 μM, 15 min) WIL-2 cells, 3.7 × 10⁷ cells per ml, were pulse labeled for 40 (*A* and *D*), 80 (*B* and *E*), and 180 (*C* and *F*) sec with 100 μCi (4.13 nmol) of [³H]deoxyuridine. At the end of the pulse labeling periods, cells were harvested, extracted with NaDodSO₄, and analyzed by alkaline sucrose gradient centrifugation as described in *Materials and Methods*, except that the precipitated cells from the methanol extractions were subsequently washed four times with 60% methanol to remove unincorporated nucleoside from the cell samples before cell disruption and centrifugation.
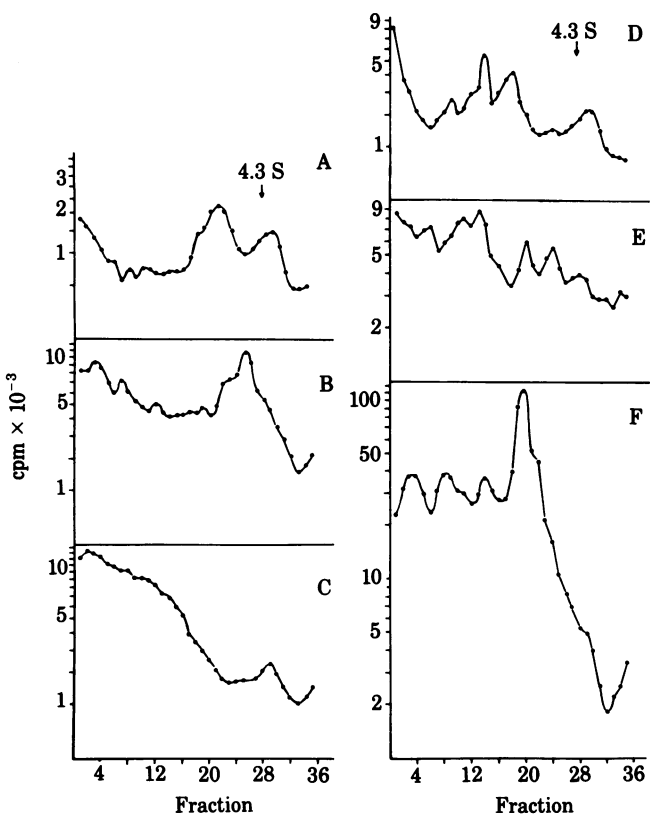
FIG. 5. Alkaline sucrose gradient analysis of cells pulse labeled with [³H]thymidine in the presence and absence of DDMP. WIL-2 cells, 1 × 10⁷ per ml, were pulse labeled with 0.5 μM [³H]thymidine for 30 sec (*A*), 120 sec (*B*), and 300 sec (*C*), and after 15 min of exposure to 5 μM DDMP for the same time periods in *D, E,* and *F*, respectively. DNA was then extracted and analyzed by alkaline sucrose gradients.

DNA or degradation of the DNA, with a concomitant accumulation of a low molecular weight DNA fraction.

DISCUSSION

Inhibition of dTMP synthesis by antifolates in human lymphoblastoid cells not only reduced the amount of labeled deoxyuridine that was incorporated into DNA but also led to abnormal DNA synthesis in drug-treated cells. Residual DNA synthesis in DDMP-treated human lymphoblastoid cells progressed by substitution of dUMP for dTMP when exogenous labeled deoxyuridine was provided as a DNA precursor. In the presence of DDMP, accumulation of DNA larger than 4 S Okazaki fragments was preferentially inhibited during 40- to 180-sec periods of labeling with [^{3}H]deoxyuridine. Small fragments of DNA similar to those seen with bacterial deoxyuridine triphosphatase-deficient *(sof)* mutants (15–17) were also usually observed under these conditions. However, the greatly inhibited levels of incorporation in the presence of DDMP precluded unambiguous demonstration of their synthesis. Moreover, the distribution of DNA labeled with thymidine in DDMP-treated cells was skewed towards intermediate fragments larger than 4 S but smaller than control DNA. It was not clear from these experiments, however, whether this DNA arose from a block to DNA synthesis leading to accumulation of intermediate-sized DNA, or from fragmentation of newly synthesized high molecular weight DNA. Although experiments in permeabilized cells and *in vitro* extracts have shown that mammalian cells have an enzymatic mechanism capable of misincorporating deoxyuridine into DNA and subsequently removing uracil (18, 19), the present studies go beyond this to document a possible *in vivo* lesion in DNA synthesis coincident with antifolate-induced misincorporation of deoxyuridine in eukaryotes. DNA fragmentation has been previously reported by others as a consequence of thymidylate synthetase inhibition (14); misincorporation of dUMP could also provide at least part of the explanation for this observation.

Direct evidence for the mechanism by which the DNA labeling patterns observed in alkaline sucrose gradients arise has not yet been obtained in our studies. Substitution of dUMP for dTMP could result in depyrimidation by deoxyuridine-specific *N*-glycosylase activity or in temporary damage to newly synthesized DNA as a consequence of other aspects of enzymatic repair mechanisms. Both depyrimidation and nuclease-dependent lesions could lead to apparent fragmentation of newly synthesized DNA as determined by alkaline sucrose gradient analysis. Moreover, except for the thymidine pulse labeling experiments in DDMP-treated cells described above, most of the experiments in our studies employed nonphysiologic concentrations of exogenous deoxyuridine to promote misincorporation of dUMP into DNA. However, the time-dependent increase in intermediate-sized DNA observed in DDMP-treated (5 μM) cells, which were labeled with thymidine without deoxyuridine is consistent with repair-induced fragmentation of newly replicated DNA as a consequence of antifolate treatment alone.

The observation that antifolates cause misincorporation of dUMP into DNA adds a dimension to the complex role of intracellular dUMP levels in the differential response of cells to these drugs. Thus, dUMP accumulation may not only lead to

increased depletion of tetrahydrofolate pools as suggested by Moran *et al.* (20) but could also have its own direct and potentially toxic interaction with the DNA replication and repair mechanisms of the cell.

In conclusion, metabolic blockade of dTMP synthesis can lead to significant misincorporation of dUMP and to abnormal DNA synthesis. The observation of dUMP misincorporation described above is consistent with the report of Goulian *et al.* (21) in MTX-treated cells that appeared while this manuscript was being prepared. DDMP, however, offers the substantial advantage for study of DNA synthesis in human lymphoblastoid cells of achieving a steady-state of inhibition within 5 sec of its addition to the culture growth medium (22). This obviates the need for pretreatment of cells with drug to establish a steady-state of inhibition. Moreover, because intracellular levels of DDMP are directly controlled by exogenous drug concentrations, study of antifolate-induced effects are facilitated at different inhibition levels without complication of a concentrative active transport system. The potential for study of sequential biochemical effects of DDMP on nucleotide precursor pools for DNA and DNA synthesis, is, therefore, greater with DDMP than with transport dependent drugs such as MTX. The *in vivo* significance of deoxyuridine misincorporation, however, is still unknown.

1. Tye, B.-K. & Lehman, I. R. (1977) *J. Mol. Biol.* 117, 293–306.
2. Arima, T., Akiyoshi, H. & Fujii, S. (1977) *Cancer Res.* 37, 1598–1601.
3. Schlomai, J. & Kornberg, A. (1978) *J. Biol. Chem.* 253, 3305–3312.
4. Williams, M. V. & Cheng, Y.-C. (1979) *J. Biol. Chem.* 254, 2897–2901.
5. Friedberg, E. C., Ganesan, A. J. & Minton, K. (1975) *J. Virol.* 16, 315–321.
6. Kuhnlein, U., Lee, B. & Linn, S. (1978) *Nucleic Acids Res.* 5, 117–125.
7. Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B. & Sperens, B. (1977) *J. Biol. Chem.* 252, 3286–3294.
8. Sirover, M. A. (1979) *Cancer Res.* 39, 2090–2095.
9. Sekiguchi, M., Hayakawa, H., Makino, F., Tanaka, K. & Okada, Y. (1976) *Biochem. Biophys. Res. Commun.* 73, 293–299.
10. Sedwick, W. D., Brown, O. E., Frazer, T., Kutler, M. & Laszlo, J. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 37, 1613 (abstr.).
11. Sedwick, W. D., Kutler, M., Frazer, T., Brown, O. E. & Laszlo, J. (1979) *Cancer Res.* 39, 3612–3618.
12. Wang, T. S.-F., Sedwick, W. D. & Korn, D. (1974) *J. Biol. Chem.* 249, 841–850.
13. Kidson, C. (1966) *J. Mol. Biol.* 17, 1–9.
14. Perlman, D. & Huberman, J. A. (1977) *Cell* 12, 1029–1043.
15. Tye, B.-K., Nyman, P.-O., Lehman, I. R., Hochhauser, S. & Weiss, B. (1977) *Proc. Natl. Acad. Sci. USA* 74, 154–157.
16. Tye, B.-K., Chien, J., Lehman, I. R., Duncan, B. K. & Huber, R. W. (1978) *Proc. Natl. Acad. Sci. USA* 75, 233–237.
17. Olivera, B. M. (1978) *Proc. Natl. Acad. Sci. USA* 75, 238–242.
18. Wist, E., Unhjem, O. & Krokan, H. (1978) *Biochim. Biophys. Acta* 520, 253–270.
19. Grafstrom, R. H., Tseng, B. Y. & Goulian, M. (1978) *Cell* 15, 131–140.
20. Moran, R. I., Mulkins, M. & Heidelberger, C. (1979) *Proc. Natl. Acad. Sci. USA* 76, 5924–5928.
21. Goulian, M., Bleile, B. & Tseng, B. Y. (1980) *Proc. Natl. Acad. Sci. USA* 77, 1956–1960.
22. Sedwick, W. D., Fyfe, M. J., Brown, O. E., Frazer, T. A., Kutler, M. & Laszlo, J. (1979) *Mol. Pharmacol.* 16, 110–116.