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## Articles

# UV-Induced Pyrimidine Hydrates in DNA Are Repaired by Bacterial and Mammalian DNA Glycosylase Activities†

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ABSTRACT: *Escherichia coli* endonuclease III and mammalian repair enzymes cleave UV-irradiated DNA at AP sites formed by the removal of cytosine photoproducts by the DNA glycosylase activity of these enzymes. Poly(dG-[$^3$H]dC) was UV irradiated and incubated with purified endonuclease III. $^3$H-Containing material was released in a fashion consistent with Michaelis-Menten kinetics. This $^3$H material was determined to be cytosine by chromatography in two independent systems and microderivatization. $^3$H-Containing material was not released from nonirradiated copolymer. When poly(dA-[$^3$H]dU) was UV irradiated, endonuclease III released $^3$H-containing material that coeluted with uracil hydrate (6-hydroxy-5,6-dihydrouracil). Similar results are obtained by using extracts of HeLa cells. There results indicate that the modified cytosine residue recognized by endonuclease III and the mammalian enzyme is cytosine hydrate (6-hydroxy-5,6-dihydrocytosine). Once released from DNA through DNA-glycosylase action, the compound eliminates water, reverting to cytosine. This is consistent with the known instability of cytosine hydrate. The repairability of cytosine hydrate in DNA suggests that it is stable in DNA and potentially genotoxic.

Endonuclease III of *Escherichia coli* incises DNA damaged by ionizing and UV radiation and oxidizing agents such as osmium tetraoxide (Radman, 1976; Gates & Linn, 1977; Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984; Weiss & Duker, 1986, 1987; Doetsch et al., 1987). Analogous enzyme activities have been identified in yeast and mammalian cells and tissues [Breimer, 1983; Doetsch et al., 1986; Higgins et al., 1987; Lee et al., 1987; Gossett et al., 1988; reviewed in Wallace (1988)]. These enzymes contain two activities that act sequentially: a DNA glycosylase activity, which releases the modified DNA base, and an AP endonuclease activity, which cleaves the DNA backbone at abasic sites (Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984). These enzymes release modified thymine residues from DNA exposed to ionizing radiation or osmium tetraoxide (Breimer, 1983; Doetsch et al., 1986; Higgins et al., 1987). The common structural feature of the base modifications recognized by these enzymes is loss of aromaticity of the thymine ring. Subsequently, it has been proposed that enzymatic release is mediated via a transiminization reaction between the ring open form of the modified thymine residue and an enzyme amino group (Kow & Wallace, 1987; Bailly & Verly, 1987).

In contrast to incision at thymine residues after exposure of DNA to ionizing radiation, endonuclease III and the analogous mammalian enzyme activity incise UV-irradiated DNA at cytosine residues, releasing cytosine-derived material (Doetsch et al., 1986; Weiss & Duker, 1986, 1987). The chemical nature of this repairable modified cytosine residue has not been characterized to date. In the experiments described in the succeeding sections, we used UV-irradiated poly(dG-[$^3$H]dC) to demonstrate that the modified cytosine residue is cytosine photohydrate.

## Experimental Procedures

### *Materials*

*Enzyme.* Endonuclease III was purified from the cloned *E. coli nth* gene (Asahara et al., 1989). The enzyme was stored in a 1 mg/mL solution (100 mM potassium phosphate, pH 6.6, 50% glycerol). Enzyme was diluted for enzyme assays with the following buffer: 50 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 0.5 mg/mL molecular grade BSA (from BRL), and 10% glycerol.

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*Radionucleotides and Bases.* [5-$^3$H]-2′-Deoxycytidine 5′-triphosphate (dCTP) (28.2 Ci/mmol) was purchased from NEN. [5-$^3$H]-2′-Deoxyuridine 5′-triphosphate (dUTP) (28.2 Ci/mmol) was purchased from Amersham. [2-$^{14}$C]Cytosine (58 mCi/mmol) and [2-$^{14}$C]uracil (56 mCi/mmol) were purchased from Moravek Biochemicals.

*Uracil Hydrate.* 6-Hydroxy-5,6-dihydrouracil (uracil hydrate) was synthesized by irradiation of an aqueous 1 mM uracil solution with a high-intensity UV lamp (Rayonet photochemical reactor, Southern New England Co., Hamden, CT, using 16 RP-2537 angstrom bulbs) for 16 h. The compound was separated by repetitive HPLC on C18 ODS columns to produce a single peak by HPLC and a single spot by TLC. The hydrate was detected in the HPLC effluent with a refractometer. The compound was visualized on silica gel 60 F-254 plates (Merck) by heating the plate at greater than 180 °C for 15 min to convert the uracil hydrate to uracil. The structure of this compound was confirmed by mass spectroscopy and nuclear magnetic resonance spectroscopy.

(*a*) *$^1$H Nuclear Magnetic Resonance Measurements.* The $^1$H NMR spectra were recorded at 200.13 MHz (Bruker AM 200 spectrometer) in hexadeuterated dimethylsulfoxide (DMSO-$d_6$) at 20 °C. Tetramethylsilane (TMS) was used as an internal reference. Assignment of the protons is on the basis of specific decoupling experiments and chemical exchange of N-1, N-3, and hydroxyl protons with $D_2O$. 200.13 MHz $^1$H NMR (DMSO-$d_6$, TMS) (ppm): 2.31 (ddd, 1 H, H-5e, $J_{5e,5a}$ = −16.5 Hz, $J_{5e,6}$ = 1.9 Hz, $J_{5e,3}$ = 1.3 Hz), 2.78 (dd, 1 H, H-5a, $J_{5a,5e}$ = −16.5 Hz, $J_{5a,6}$ = 4.3 Hz), 4.84 (ddd, 1 H, H-6, $J_{5a,5b}$ = −16.5 Hz, $J_{6,5a}$ = 4.3 Hz, $J_{6,5e}$ = 1.9 Hz, $J_{6,1}$ = 4.2 Hz), 8.11 (br d, 1 H, H-1, $J_{1,6}$ = 4.2 Hz), 10.07 (br s, 1 H, H-3).

(*b*) *Mass Spectrometry Experiments.* Positive-ion fast atom bombardment (FAB) mass spectra (glycerol matrix) were obtained on a Kratos spectrometer (Model MS 50) equipped with a FAB gun (8-keV xenon atoms). FAB-MS, *m/z* (rel intensity), positive mode: 223 (10, $[M + \text{glycerol}]^+$), 131 (11, $[M + H]^+$), 113 (8, $[M - H_2O + H]^+$).

## Methods

*Synthesis of Poly(dG-[$^3$H]dC) and Poly(dA-[$^3$H]dU).* Poly(dG-[$^3$H]dC) and poly(dA-[$^3$H]dU) were synthesized from poly(dG-dC) and poly(dA-dU), respectively, by using a nick translation kit (NEN, NEK-004Z). Each translation reaction was conducted by using 10 μCi of [$^3$H]dCTP or [$^3$H]dUTP and 0.5 μg of poly(dG-dC) or poly(dA-dU) (Pharmacia). Reaction mixtures were incubated for 2 h at 14 °C. Reactions were stopped by adding 70 μL of 0.03 M EDTA. The samples were incubated for 15 min at 70 °C and then cooled over a period of 2 h to room temperature. Translated DNA was separated from unincorporated radioactive material by using a Sephadex G-50 minicolumn (Pharmacia) with a 10 mM Tris-HCl elution buffer, pH 7.5. To precipitate the DNA, sodium acetate was added to a final concentration of 0.3 M, and 2.5 volumes of cold ethanol was added. All reactions and precipitations were carried out in siliconized tubes. After the DNA was pelleted by centrifugation, the supernatant was decanted and the DNA was dried under vacuum and resuspended in TE buffer (0.01 M Tris, 0.001 M EDTA, pH 8.0). The specific activity of the translated DNA was typically 10–12 × $10^6$ dpm/μg.

*UV Irradiation.* Samples were irradiated (Carrier & Setlow, 1971; Duker & Teebor, 1975) by use of a double fixture containing two General Electric 15-W germicidal bulbs (G15TA). The primary output of these bulbs occurs at 254 nm. These bulbs delivered a combined dose of 10 kJ/m$^2$ in 6 min at a distance of 13.5 cm. Irradiation of DNA was carried out in weighing boats on ice.

*Confirmation of Endonuclease III Endonuclease Activity.* To determine that endonuclease III was active in an endonuclease assay, 2 μg of pBR322 plasmid DNA was exposed to approximately 12 kJ of UV radiation in 12 μL of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Following irradiation, 0.75 μg of DNA was incubated with 1 ng of endonuclease III in a total volume of 10 μL. Three control reactions were also performed as follows: (a) irradiated DNA without enzyme; (b) unirradiated DNA with enzyme; and (c) unirradiated DNA without enzyme. Enzyme diluent was substituted for enzyme solution in enzyme-free controls. The final reaction mixture contained 50 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 0.1 mg/mL BSA, and 0.75 μg of DNA. Reactions were conducted in siliconized tubes. After 30 min of incubation at 37 °C, reaction tubes were cooled on ice. The reaction mixtures were then analyzed on a 0.7% agarose gel containing ethidium bromide. One nanogram of endonuclease III completely converted the UV-irradiated supercoiled pBR322 DNA to the nicked form. Controls did not convert the supercoiled DNA.

*Assay for the Enzymatic Release of $^3$H-Containing Material from Poly(dG-[$^3$H]dC) or Poly(dA-[$^3$H]dU).* Poly(dG-[$^3$H]dC) or poly(dA-[$^3$H]dU) was irradiated and 50 ng (5.0–6.0 × $10^5$ dpm) of copolymer was incubated with indicated amounts of endonuclease III. Enzyme incubations were carrier out in a volume of 12 μL for 30 min at 37 °C under buffer conditions identical with those used for pBR322 endonuclease assay. The reaction was stopped by addition of 12 μL of BSA (10 mg/mL) and 50 μL of cold acetone, unless otherwise noted. After incubation overnight at −20 °C, acetone-insoluble material was centrifuged at 1200*g* for 15 min. The supernatant fluid was decanted and dried under vacuum. Samples were redissolved in 200 μL of water and analyzed by HPLC or TLC.

*HPLC Analysis of Enzymatically Released $^3$H-Containing Material from Poly(dG-[$^3$H]dC) or Poly(dA-[$^3$H]dU).* Samples were analyzed on a 5-μm Ultrasphere ODS column (Altex; 0.46 × 25 cm). Ammonium formate (50 mM) was used as an eluant at a rate of 1 mL/min. The column was washed for 15 min with methanol after each run and reequilibrated in ammonium formate for 20 min prior to the injection of the next sample. All samples were monitored through the use of 10 μL of 1 mM uracil and cytosine as UV markers. The retention time of added nonradioactive marker uracil hydrate was determined by collecting HPLC fractions, drying them, and analyzing them by TLC as described below. The elution of radioactivity was monitored, as indicated, either through the use of a Radiomatic Flo-One in-line radioactivity detector or through the collection of 1-min (1-mL) fractions from the column for liquid scintillation counting. For further analysis of radioactive peaks separated by HPLC, fractions containing radioactive material were lyophilized and rechromatographed as indicated.

*Thin-Layer Chromatography (TLC).* Samples separated by HPLC were dissolved in methanol and spotted onto 10 cm × 10 cm silica gel plates (plastic-backed plates; silica gel 60 F-254) with a spacing of 1.5 cm between samples. The chromatographs were run with the lower layer of chloroform:methanol:$H_2O$ (4:2:1) to which 5 mL of methanol had been added for each 100 mL of organic phase (Teoule et al., 1974; Cadet et al., 1976) until the solvent front reached 1 cm from the top of the plate. Plates were air-dried and then heated for 15 min at 180 °C. Heating transforms uracil hydrate into

uracil (Kochetkov & Budovskii, 1972; Fisher & Johns, 1976), which becomes detectable by UV light. Following chromatography, regions coeluting with UV fluorescent markers were outlined. The silica gel from these regions and intervening regions was scraped into scintillation vials, to which 1 mL of $H_2O$ was added. Radioactivity was then analyzed by liquid scintillation counting.

*Derivatization of $^3$H-Containing Material Released from Poly(dG-[$^3$H]dC) by Endonuclease III.* [$^{14}$C]Cytosine (500 dpm) was added to the acetone-soluble products of the reaction of endonuclease III and poly(dG-[$^3$H]dC). Samples of acetone-soluble material released by endonuclease III were purified initially by HPLC as above. The fractions containing the $^3$H and $^{14}$C radioactivity were then derivatized by techniques described previously (Frenkel et al., 1985; Boorstein et al., 1987). Briefly, the fractions were dried and redissolved in dry pyridine. Acetic anhydride was added, and the solution was incubated overnight at room temperature. The reaction was terminated by the addition of 500 $\mu$L of water. The sample was then dried, resuspended in 5% methanol:95% 0.05 M ammonium formate (v/v), and chromatographed as above by using a 20-min, 3%–60% gradient of methanol/0.05 M ammonium formate, pH 6.5, as eluant. The radioactivity in 1-mL fractions as then determined by liquid scintillation counting.

*Preparation and Enzymatic Assay of HeLa Cell Sonicates.* HeLa cells were maintained and grown in 150-cm$^2$ flasks as described previously (Higgins et al., 1987; Boorstein et al., 1987). Two flasks containing cells in log phase growth were washed twice with 15 mL of PBS and removed from the plate with 20 mL of saline EDTA. Suspensions were centrifuged at 3000 rpm for 10 min and washed three times with 15 mL of Hanks balanced salt solution. Approximately 2.2 × 10$^7$ cells were resuspended in 1 mL of TKE (50 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM EDTA) and sonicated. DTT was added to 0.5 mM, and the sonicate was centrifuged at 8000 rpm for 10 min. The supernatant was decanted and assayed.

HeLa cell extracts were assayed for their ability to remove $^3$H-containing material from poly(dG-[$^3$H]dC) by modifying the endonuclease III scheme described above. Each reaction mixture contained 100 rather than 50 ng of poly(dG-[$^3$H]dC) and 15 $\mu$L of diluted enzyme. Reaction tubes were incubated for 2 h at 37 °C. Reactions were stopped by adding 12 $\mu$L of BSA (10 mg/mL) and 75 $\mu$L of acetone. Tubes were refrigerated overnight and centrifuged. The supernatant as recovered, dried under vaccum, and redissolved in 200 $\mu$L of $H_2O$. Samples were analyzed on HPLC with added UV markers and counted by in-line radioactive flow detection.

## Results

*Characterization of the Chemical Identity of $^3$H-Containing Material Released from Poly(dG-[$^3$H]dC).* Poly(dG-[$^3$H]dC) was irradiated with 100 kJ/m$^2$ of 254-nm UV light and then incubated with 1 ng of endonuclease III. The acetone-soluble products of this reaction were then analyzed by HPLC. Greater than 90% of the recovered radioactivity coeluted with cytosine, which had been added to the sample prior to HPLC analysis (Figure 1). In this HPLC system, uracil hydrate, cytosine, and uracil eluted at 4.2, 5.2, and 6.0 min, respectively. In the absence of irradiation, or in the absence of enzyme, no significant amounts of radioactive material were released (Figure 1). There was also no release of radioactive material from irradiated poly(dG-[$^3$H]dC) following incubation with boiled enzyme.

Because the recovery of cytosine as the product of this enzymatic reaction was such a surprising result, the chemical

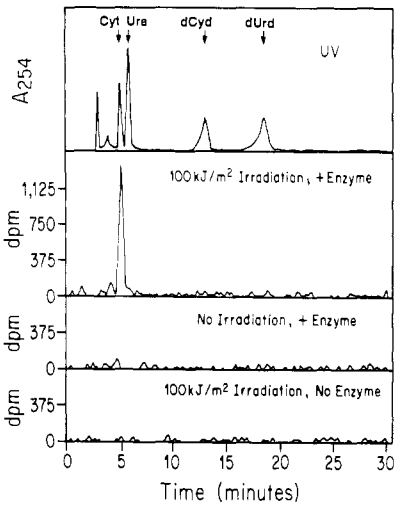


FIGURE 1: HPLC profile of acetone-soluble products released from poly(dG-[$^3$H]dC) by endonuclease III. Endonuclease III was incubated with poly(dG-[$^3$H]dC) for 30 min as described in the text. The top panel shows the $A_{254}$ of the eluant ($H_2O$, 1 mL/min) to which Cyt, Ura, dCyd, and dUrd were added as nonradioactive markers, as indicated in the figure. The second panel shows the radioactivity released by incubation of enzyme with substrate irradiated with 100 kJ/m$^2$. The third panel shows the radioactivity released by incubation of enzyme with unirradiated substrate. The bottom panel shows the release of radioactivity from irradiated substrate incubated without enzyme.

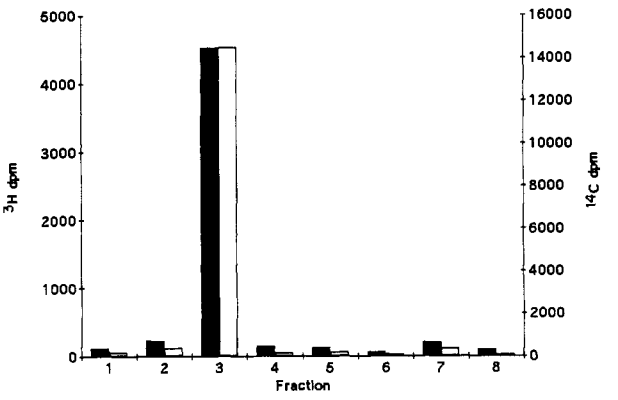


FIGURE 2: TLC of acetone-soluble products released from poly(dG-[$^3$H]dC) by endonuclease III. $^3$H-containing material released from poly(dG-[$^3$H]dC) by endonuclease III was isolated by HPLC and analyzed by TLC. Marker [$^{14}$C]cytosine was analyzed in a parallel lane. Radioactivity was determined for eight fractions corresponding with the following $R_f$s: fraction 1, 0–0.04; fraction 2, 0.04–0.10; fraction 3, 0.10–0.21; fraction 4, 0.21–0.29; fraction 5, 0.29–0.34; fraction 6, 0.34–0.42; fraction 7, 0.42–0.49; fraction 8, 0.49–0.72. Cytosine, uracil, and uracil hydrate coeluted with fractions 3, 5, and 7, respectively, in this analysis. $^3$H radioactivity (black bars, left axis); $^{14}$C radioactivity (open bars, right axis).

identity of the $^3$H-containing material coeluting with cytosine was confirmed in additional independent chromatographic systems. First, a sample identical with that shown in Figure 1 was separated by HPLC as in Figure 1. The HPLC fraction containing the $^3$H-containing material was dried and analyzed by TLC. In this chromatographic system, using chloroform:methanol:$H_2O$ as a mobile phase, cytosine, uracil hydrate, and uracil had $R_f$ values of 0.16, 0.31, and 0.46, respectively. All the enzymatically released material coeluted with UV marker cytosine. In addition, marker [$^{14}$C]cytosine chromatographed in a parallel lane on the same plate had an identical $R_f$ (Figure 2).

To further confirm that the $^3$H-containing material coeluting with cytosine was authentic [$^3$H]cytosine, a sample of this material was collected and reacted with acetic anhydride to

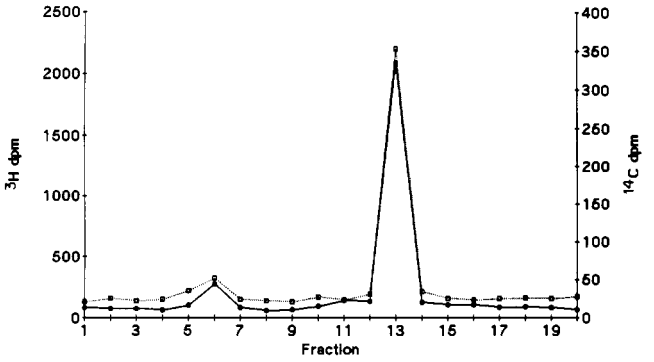


FIGURE 3: Derivatization of $^3$H-containing material released from poly(dG-[$^3$H]dC) by endonuclease III. A sample of $^3$H-containing material to which marker [$^{14}$C]cytosine had been added was derivatized and analyzed by HPLC as described in the text.

form acetylated derivatives. [$^{14}$C]Cytosine was added to the sample prior to the initial HPLC separation. Enzymatically released [$^3$H]cytosine and added [$^{14}$C]cytosine were converted to cytosine $N^4$-acetate to exactly the same degree. In the HPLC system used in this analysis, a 20-min 3–60% methanol gradient was used to elute the cytosine acetate from the HPLC column. Cytosine eluted at fraction 6, while the elution of cytosine acetate occurred at fraction 13 (Figure 3).

These experiments unambiguously demonstrated that cytosine was the principal product recovered from the reaction of endonuclease III with irradiated poly(dG-[$^3$H]dC). Our experiments rigorously excluded the possibility that cytosine itself in unmodified copolymer was a substrate for endonuclease III, since no release was seen when the copolymer was not irradiated.

*Characterization of the Chemical Identity of $^3$H-Containing Material Released from Poly(dA-[$^3$H]dU).* Poly(dA-[$^3$H]dU) was prepared as a substrate for endonuclease III analogous to poly(dG-[$^3$H]dC). Poly(dA-[$^3$H]dU) was irradiated with 10 kJ/m$^2$ of 254-nm UV light and then incubated with 1 ng of endonuclease III. The acetone-soluble products of this reaction were then analyzed by HPLC. Greater than 90% of the recovered radioactivity eluted with a retention time corresponding to the retention time of uracil hydrate (Figure 4). In the absence of irradiation, or in the absence of enzyme, no significant amounts of radioactive material were released (Figure 4).

The chemical identity of the $^3$H-containing materal coeluting with uracil hydrate was confirmed in additional independent chromatographic systems. First, a sample identical with that shown in Figure 4 was separated by HPLC as in Figure 4. The HPLC fraction containing the $^3$H-containing material was dried, divided in two, and analyzed by TLC. Half the sample was spotted immediately. The other half was heated for 60 min at 95 °C. These conditions completely converted uracil hydrate to uracil. The uracil hydrate had an $R_f$ of 0.42 and was not visible under UV light until the plate was heated, while uracil was visible before heating and had an $R_f$ of 0.56. Before heating, all the enzymatically released $^3$H-containing material coeluted with uracil hydrate. After heating, the $^3$H-containing material coeluted with uracil (Figure 5). These results unambiguously demonstrated enzymatic release of uracil hydrate from UV-irradiated poly(dA-[$^3$H]dU) by endonuclease III.

*Kinetics of Release.* One nanogram of endonuclease III was incubated with poly(dG-[$^3$H]dC) that had been irradiated with increasing doses of far-UV light, and [$^3$H]cytosine release was measured. Over the range of irradiation used, the concentration of the modified base in DNA was assumed to increase directly with the amount of energy input. Thus, the amount

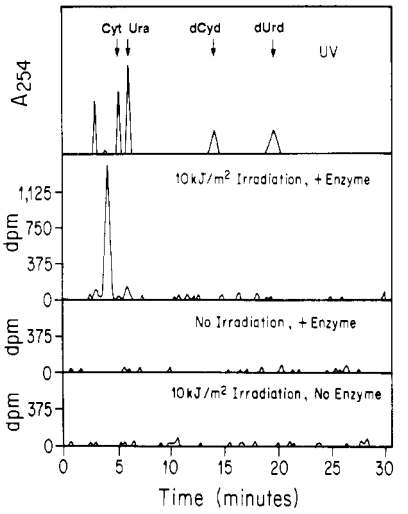


FIGURE 4: HPLC profile of acetone-soluble products released from poly(dA-[$^3$H]dU) by endonuclease III. Endonuclease III was incubated with poly(dA-[$^3$H]dU) for 30 min as described in the text. The top panel shows the $A_{254}$ of the eluant ($H_2O$, 1 mL/min) to which Cyt, Ura, dCyd, and dUrd were added as nonradioactive markers, as indicated in the figure. The second panel shows the radioactivity released by incubation of enzyme with substrate irradiated with 10 kJ/m$^2$. The third panel shows the radioactivity released by incubation of enzyme with unirradiated substrate. The bottom panel shows the release of radioactivity from irradiated substrate incubated without enzyme.

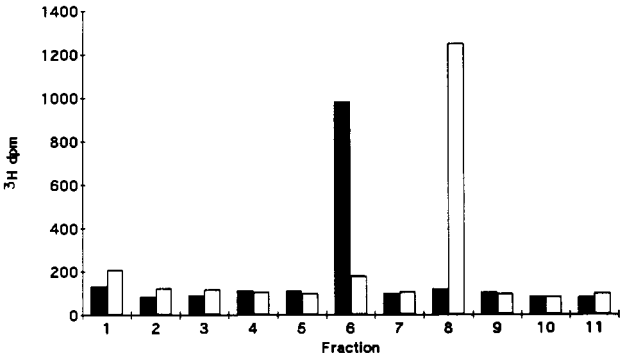


FIGURE 5: TLC of acetone-soluble products released form poly(dA-[$^3$H]dU) by endonuclease III. $^3$H-containing material released from poly(dA-[$^3$H]dU) by endonuclease III was isolated by HPLC and analyzed by TLC. Half of the sample was heated prior to analysis. Radioactivity was determined for 11 fractions corresponding to the following $R_f$s: fraction 1, 0–0.03; fraction 2, 0.03–0.14; fraction 3, 0.14–0.22; fraction 4, 0.22–0.32; fraction 5, 0.32–0.38; fraction 6, 0.38–0.45; fraction 7, 0.45–0.55; fraction 8, 0.55–0.64; fraction 9, 0.64–0.71; fraction 10, 0.71–0.78; fraction 11, 0.78–0.95. Uracil was visualized at fraction 8, while uracil hydrate was visualized at fraction 6, after heating. Cytosine coeluted with fraction 3 in this analysis. $^3$H radioactivity before heating (black bars); $^3$H radioactivity after heating (open bars).

of UV light input was used as a substitute measure for the concentration of substrate in DNA. Over the 0–800 kJ/m$^2$ dose range examined, release of [$^3$H]cytosine followed $v$ vs [S] kinetics (Figure 6). At the dose that produced the greatest amount of release, 800 kJ/m$^2$, approximately 1% of the original substrate radioactivity was released as cytosine.

The incubation of irradiated poly(dA-[$^3$H]dU) with endonuclease III resulted in greater release of uracil hydrate as compared to cytosine release from irradiated poly(dG-[$^3$H]dC). At 30 kJ/m$^2$, approximately 9% of the total original substrate radioactivity was recovered as the uracil hydrate (Figure 7). At higher doses of radiation, the rate of release decreased, suggesting that poly(dA-[$^3$H]dU) was destroyed by higher doses of radiation. This was probably due to alteration of the

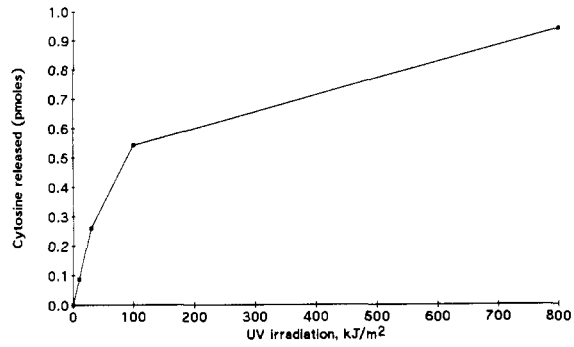


FIGURE 6: $v$ vs [S] plot of cytosine release from poly(dG-[$^3$H]dC). One nanogram of endonuclease III was incubated with 50 ng of irradiated poly(dG-[$^3$H]dC), and cytosine release in 30 min was measured by HPLC.


FIGURE 7: $v$ vs [S] plot of uracil hydrate release from poly(dA-[$^3$H]dU). One nanogram of endonuclease III was incubated with 50 ng of irradiated poly(dA-[$^3$H]dU), and uracil hydrate release in 30 min was measured by HPLC.

structure of the polynucleotide as the number of modified residues increased. Endonuclease III has an absolute requirement of double-stranded DNA as substrate (Breimer, 1983; Breimer & Lindahl, 1984; Higgins et al., 1987). As the conformation of the polynucleotide is altered, the enzyme binds to it with less efficiency. When substantial amounts of the thymine residues in both strands of poly(dA-dT) were chemically oxidized to thymine glycol, the polynucleotide was no longer a substrate for endonuclease III (Higgins et al., 1987).

The increased release of pyrimidine photohydrates from poly(dA-[$^3$H]dU) as compared to release from poly(dG-[$^3$H]dC) most likely reflects the increased net production of uracil photoproducts in DNA, as compared with cytosine photoproducts. Uracil in solution was more than 10-fold more susceptible to destruction than was cytosine when exposed to UV radiation (Kochetkov & Budovskii, 1972; Fisher & Johns, 1976; unpublished results). Similar results were seen with the corresponding deoxyribonucleosides. Of course, differences in UV-induced modifications on bases or deoxyribonucleosides in solution do not necessarily reflect modification of nucleotides in copolymers. We therefore cannot completely exclude the possibility that similar amounts of photohydrates were produced in the two substrates at equivalent UV doses but that endonuclease III removed uracil hydrate with greater efficiency.

To determine the velocity of endonuclease III mediated release of pyrimidine from irradiated substrates, irradiated poly(dG-[$^3$H]dC) was incubated with increasing amounts of enzyme. The radiation dose for this experiment was chosen to be about twice the apparent $K_m$ as determined in Figure 6. Higher doses of radiation were not used in this experiment to avoid photochemical degradation of the polynucleotide, rendering it unsuitable as an enzyme substrate. The $v$ vs [E]$_t$ plot is shown in Figure 8. The specific activity was determined from the initial portion of this curve to be 0.044 pmol of cytosine released (ng of protein)$^{-1}$ min$^{-1}$. Similarly, the kinetics of uracil hydrate released from poly(dA-[$^3$H]dU) were determined, and the corresponding $v$ vs [E]$_t$ plot is also shown in Figure 8. The specific activity, as calculated from the initial portion of this curve, was 0.35 pmol of uracil hydrate released (ng of protein)$^{-1}$ min$^{-1}$.

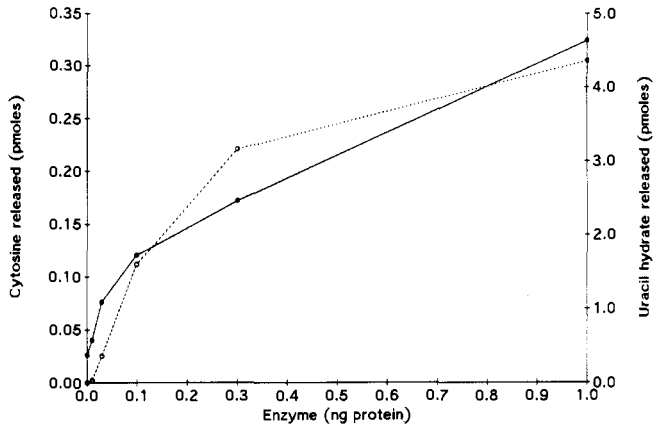


FIGURE 8: $v$ vs [E]$_t$ plots of cytosine release from poly(dG-[$^3$H]dC) and of uracil hydrate release from poly(dA-[$^3$H]dU). Increasing amounts of endonuclease III were incubated with 50 ng of 100 kJ/m$^2$ irradiated poly(dG-[$^3$H]dC), and cytosine release in 30 min was measured by HPLC (solid symbols). Increasing amounts of endonuclease III were incubated with 50 ng of 10 kJ/m$^2$ irradiated poly(dA-[$^3$H]dU), and uracil hydrate release in 30 min was measured (open symbols).

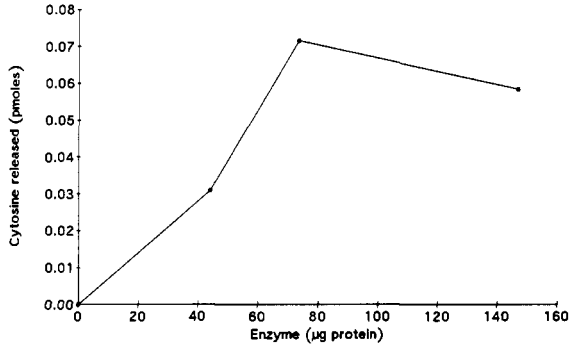


FIGURE 9: $v$ vs [E]$_t$ plot of cytosine release from poly(dG-$^3$H]dC) with HeLa cell sonicate. Increasing amounts of HeLa cell sonicate were incubated with 100 ng of 100 kJ/m$^2$ irradiated poly(dG-[$^3$H]dC), and cytosine release in 120 min was measured by HPLC.

*Activity of HeLa Cell Sonicates against Poly(dG-[$^3$H]dC).* Increasing concentrations of HeLa cell sonicates were incubated with poly(dG-[$^3$H]dC), and the acetone-soluble material was analyzed by HPLC. As with the bacterial enzyme, radioactivity was recovered only coeluting with cytosine. Figure 9 shows the $v$ vs [E]$_t$ plot for this experiment. No release was seen when substrate was incubated with boiled enzyme. The specific activity as determined from the initial portion of this curve was 7 fmol (mg of protein)$^{-1}$ min$^{-1}$. This compares with a specific activity of 22 fmol mg$^{-1}$ min$^{-1}$ for the release of thymine glycol from chemically oxidized poly(dA-dT) by a similar HeLa cell preparation (Higgins et al., 1987).

## Discussion

We have determined that the $^3$H-containing material enzymatically released from UV-irradiated poly(dG-[$^3$H]dC) by endonuclease III or extracts of HeLa cells is cytosine. We initially hypothesized two mechanisms to explain this result. The first was that the photochemically modified cytosine

residue reverted to cytosine through the action of the repair enzyme itself. The second was that the product released by the repair enzyme was a modified cytosine residue which reverted to cytosine during the isolation procedure prior to HPLC analysis.

Therefore, we synthesized poly(dA-[$^3$H]dU) exposed it to UV radiation. Since we had synthesized uracil hydrate, we could determine whether uracil or uracil hydrate was enzymatically released from the copolymer. If uracil had been released, this result would have indicated that during the enzymatically mediated hydrolysis of the photochemically modified uracil residue water had been eliminated. However, the recovery of uracil hydrate indicated that the enzymes recognized photohydrates in DNA and released them intact from the UV-irradiated polynucleotide backbone. We can therefore conclude that the compound recognized in UV-irradiated poly(dG-[$^3$H]dC) by the enzymes is 6-hydroxy-5,6-dihydrocytosine (cytosine hydrate). This compound is the analogous compound to cytosine that uracil hydrate is to uracil. Cytosine hydrate is highly unstable and reverts to cytosine in neutral aqueous solution (Kochetkov & Budovskii, 1972; Fisher & Johns, 1976). Uracil hydrate is relatively stable in solution, even at 37 °C, and only reverts to uracil when subjected to extremes of heat or pH (Kochetkov & Budovskii, 1972; Fisher & Johns, 1976).

Our proposal that the principal cytosine photoproduct recognized by endonuclease III and its mammalian activity is cytosine hydrate, while the principal uracil photoproduct is uracil hydrate, is consistent with the known mechanism of action of endonuclease III and related mammalian enzymes (Kow & Wallace, 1987; Bailly & Verly, 1987). These enzymes recognize modifications of pyrimidines such as thymine glycol, urea, and 5-hydroxy-5-methylhydantoin (Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984). All these modifications have an intact N1–C2–N3 backbone, but have lost the C5–C6 double bond. Uracil hydrate and cytosine hydrate are similar to these modified pyrimidines and pyrimidine fragments in this regard.

We cannot exclude the possibility that a small amount of uracil hydrate was formed in poly(dG-[$^3$H]dC) following UV irradiation and was removed from DNA by endonuclease III. A small amount of enzymatically released $^3$H-containing material eluted ahead of cytosine with a retention time similar to that of uracil hydrate (Figure 1). However, less than 5% of the released $^3$H-containing material was released as putative uracil hydrate, indicating that only a small amount of cytosine hydrate in the copolymer underwent deamination.

Our results differ from the findings of Doetsch et al., who reported that endonuclease III and a related calf thymus enzyme released a product from poly(dG-[$^3$H]dC) which differed from cytosine (Doetsch et al., 1986). It is for this reaction that we confirmed out results that cytosine was released by two independent chromatographic systems and by chemical derivatization. One possible explanation for this discrepancy between our results and theirs is that we used acetone rather than ethanol to precipitate macromolecules prior to HPLC analysis. However, a direct comparison of ethanol precipitation and acetone precipitation gave us identical results (data not shown). This result demonstrated that acetone did not destroy an ethanol-stable cytosine-derived photoproduct. Doetsch et al. felt that because "cytosine photohydrates were unstable, rapidly revert back to cytosine, and are not likely to be formed under our experimental conditions" cytosine photohydrates were not the substrate for these repair enzymes (Doetsch et al., 1986).

However, our results indicate that pyrimidine photohydrates are recognized and released by the DNA glycosylase activity of endonuclease III and by corresponding mammalian activities. Our results corroborate the inferential findings of Grossman and Rodgers that cytosine photohydrates are formed in irradiated nucleic acids (Grossman & Rodgers, 1968). We suggest that cytosine hydrate residues in DNA are sufficiently stable, perhaps through base stacking interactions, to interfere with replication or transcription in a manner similar to other nonplanar modified pyrimidines, such as thymine glycol. Therefore, if unrepaired, these modified cytosine residues would prove deleterious to the organism's survival.

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**Registry No.** [5-$^3$H]dCTP, 77778-04-2; [5-$^3$H]dUTP, 77792-49-5; poly(dG-[$^3$H]dC), 121124-87-6; poly(dA-[$^3$H]dU), 121124-89-8; poly(dG-dC), 36786-90-0; poly(dA-dU), 34607-75-5; uracil hydrate, 1194-23-6; uracil, 66-22-8; endonuclease III, 60184-90-9; DNA glycosylase, 70356-40-0.

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# $O^6$-Ethylguanine Carcinogenic Lesions in DNA: An NMR Study of $O^6$etG·T Pairing in Dodecanucleotide Duplexes†

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ABSTRACT: High-resolution two-dimensional NMR studies are reported on the self-complementary d-(C1-G2-C3-O⁶etG4-A5-G6-C7-T8-T9-G10-C11-G12) duplex (designated O⁶etG·T 12-mer) containing two symmetrically related O⁶etG·T lesion sites located four base pairs in from either end of the duplex. Parallel studies were undertaken on a related sequence containing O⁶meG·T lesion sites (designated O⁶meG·T 12-mer) in order to evaluate the influence of the size of the alkyl substituent on the structure of the duplex and were undertaken on a related sequence containing G·T mismatch sites (designated G·T 12-mer duplex), which served as the control duplex. The exchangeable and nonexchangeable proton and the phosphorus nuclei have been assigned from an analysis of two-dimensional nuclear Overhauser enhancement (NOE) and correlated spectra of the O⁶etG·T 12-mer, O⁶meG·T 12-mer, and G·T 12-mer duplexes in $H_2O$ and $D_2O$ solutions. The distance connectivities observed in the NOESY spectra of the O⁶alkG·T 12-mer duplexes establish that the helix is right-handed and all of the bases adopt an anti conformation of the glycosidic torsion angle including the O⁶alkG4 and T9 bases at the lesion site. The imino proton of T9 at the O⁶alkG·T lesion sites resonates at 8.85 ppm in the O⁶etG·T 12-mer duplex and at 9.47 ppm in the O⁶meG·T 12-mer duplex. The large upfield shift of the T9 imino proton resonance at the O⁶alkG4·T9 lesion site relative to that of the same proton in the G4·T9 wobble pair (11.99 ppm) and the A4·T9 Watson–Crick pair (13.95 ppm) in related sequences establishes that the hydrogen bonding of the imino proton of T9 to O⁶alkG4 is either very weak or absent. The imino proton of T9 develops NOEs to the $CH_3$ protons of the O⁶etG and O⁶meG alkyl groups across the base pair, as well as to the imino and H5 protons of the flanking C3·G10 base pair and the imino and $CH_3$ protons of the flanking A5·T8 base pair in the O⁶alkG·T 12-mer duplexes. These observations establish that the O⁶alkG4 and T9 residues are stacked into the duplex and that the $O^6CH_3$ and $O^6CH_2CH_3$ groups of O⁶alkG4 adopt a syn orientation with respect to the $N^1$ of the alkylated guanine. The syn orientation of the $O^6$-alkyl group precludes wobble pairing at the O⁶alkG4·T9 lesion site and favors a Watson–Crick alignment of O⁶alkG4 and T9 stabilized by one short hydrogen bond between the 4-amino group of O⁶alkG4 and the 2-carbonyl oxygen of T9 in the minor groove. Since the $O^6$-alkyl group adopts a syn orientation, the separation between the $O^6$ of O⁶alkG4 and the $O^4$ of T9 in the major groove is increased, preventing the formation of a short hydrogen bond between the $N^1$ ring nitrogen of O⁶alkG4 and the imino proton of T9. Only small proton and phosphorus chemical shift differences were detected when the O⁶etG·T 12-mer and the O⁶meG·T 12-mer duplexes are compared, which reflect small conformational differences between the two duplexes. Much larger chemical shift differences are detected when the O⁶alkG·T 12-mer duplexes are compared with the G·T 12-mer duplex.

The mutagenic and carcinogenic action of *N*-nitroso compounds is in part due to the alkylation of the carbonyl groups of the purine and pyrimidine bases in DNA [reviewed by Pegg (1977), Singer (1979), and Singer and Grunberger (1983)]. The oxygen at position 6 of guanine is alkylated more often than the other exocyclic oxygen groups in DNA, and the formation of $O^6$-alkylguanine (O⁶alkG) is believed to play the most important role in *N*-nitrosamine carcinogenesis (Pegg, 1984). The alkylation of guanine in DNA leads to G·C → A·T transition mutations presumably by the mispairing of O⁶alkG with thymine (Loveless, 1969; Coulondre & Miller, 1977). A direct link between these mutations and the initiation of cancer has been suggested from animal studies (Pegg, 1984) and has been supported by the obervation that G·C → A·T transition mutations are the cause of the activation of the

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