Comparison of the Effects of UV Irradiation on 5-Methyl-Substituted and Unsubstituted Pyrimidines in Alternating Pyrimidine−Purine Sequences in DNA†

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ABSTRACT: We previously demonstrated the UV-induced formation of cytosine hydrate in DNA and its deamination product, uracil hydrate, via their release from the DNA backbone by the DNA glycosylase activity of *Escherichia coli* endonuclease III. Subsequently, endonuclease III-mediated release of thymine hydrate from UV-irradiated poly(dA−dT) was reported. Therefore, we asked whether 5-methylcytosine residues in DNA underwent photohydration and deamination to thymine hydrate in analogy to UV-induced deamination of cytosine. An alternating DNA copolymer containing 5-methylcytosine was irradiated with UVC and incubated with endonuclease III. No 5-methylcytosine hydrate was released. Instead, UV-induced nonenzymatic release of 5-methylcytosine occurred. Similarly, incubation of UV-irradiated poly(dA−dT) with endonuclease III did not release thymine hydrate; nonenzymatic release of thymine occurred. Nonenzymatic release of 5-methylpyrimidines was oxygen dependent, enhanced by ferric ion and inhibited by free radical scavengers. In contrast, photohydration of cytosine was oxygen independent, and only small amounts of cytosine were nonenzymatically released. Thus, 5-methylpyrimidine residues within alternating Pu−Py sequences in DNA do not undergo photohydration, but instead undergo cleavage of their *N*-glycosyl bonds yielding abasic (AP) sites. The inability to repair such AP sites may explain the UV sensitivity of *E. coli xthnfo* mutants, which lack AP endonuclease activity. We suggest that *N*-glycosyl bond cleavage is mediated by radical species formed via transfer of an electron from UV-excited triplet 5-methylpyrimidines to ground state oxygen and/or ferric ions.

UV irradiation of DNA results in the formation of several photoproducts, among which is the hydrate of cytosine (5,6-dihydro-6-hydroxycytosine) (Fisher & Johns, 1976a). Although formed to a lesser degree than dimeric pyrimidine photoproducts (Mitchell et al., 1991), cytosine hydrate is potentially deleterious to organisms because it pairs with thymine and cytosine rather than the complementary guanine during replication (Ono et al., 1965; Lecomte et al., 1981), and because it decomposes by deamination to uracil hydrate. Both cytosine hydrate and uracil hydrate are removed from DNA via the DNA glycosylase activity of *Escherichia coli* endonuclease III. Pyrimidine hydrate−DNA glycosylase activities have also been identified in the cells and tissues of many other species, including humans (Boorstein et al., 1989, 1990; O'Donnell et al., 1994).

Thymine has also been reported to undergo photohydration and removal from DNA via the DNA glycosylase activity of endonuclease III (Ganguly et al., 1990). This last finding, together with the results of our cytosine experiments, led us to ask whether 5-methylcytosine residues in DNA also underwent photohydration. 5-Methylcytosine is a minor base in DNA involved in gene regulation (Bird, 1986; Holliday et al., 1990; Clawson et al., 1990) and is a "hot spot" for C to T transitions (Coulondre et al., 1978). Thus, it was of interest to determine to what extent the photohydrate of 5-methylcytosine would undergo deamination to thymine hydrate via a reaction analogous to the deamination of cytosine hydrate to uracil hydrate.

The *in vitro* model system that we used was similar to the model we had previously used to demonstrate the release of the hydrates of cytosine and uracil from DNA by endonuclease III. The use of alternating Pu−Py copolymers precludes the formation of dimeric pyrimidine photoproducts that might interfere with the action of endonuclease III. In the current experiments, a radioactively labeled alternating copolymer of dG and dmC was prepared by methylating poly(dG−dC) using Sss I methylase and ^{3}H-labeled *S*-adenosyl-L-methionine. The polymer, dissolved in aqueous solution, was irradiated with 254 nm UV light and incubated with genetically engineered *E. coli* endonuclease III, and the products of the incubation were analyzed by HPLC as previously described (Boorstein et al., 1989, 1990; O'Donnell et al., 1994).

MATERIALS AND METHODS

Enzymes. *Sss* I methylase (CpG methylase) was purchased from New England Biolabs. *E. coli* endonuclease III was purified from *E. coli* λN99 (CI857) containing the *nth* gene (Asahara et al., 1989) and stored at −20 °C at a concentration of 1 mg/mL.

Radionucleotides. *S*-[*methyl*-^{3}H]Adenosyl-L-methionine (55−85 Ci/mmol) and [*methyl*-^{3}H]thymidine 5′-triphosphate

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(70−90 Ci/mmol) were purchased from DuPont. 2′-Deoxy-[5-^{3}H]cytidine 5′-triphosphate was purchased from Amersham. [2-^{14}C]Thymine (56 mCi/mmol) was purchased from ICN.

Copolymers. The alternating copolymers poly(dG−dC) and poly(dA−dT) were purchased from Pharmacia and stored in crystalline form at −20 °C. Prior to use, the copolymer was dissolved in water, and the concentration was determined by measuring the absorbance at 260 nm, assuming that 50 μg/mL = 1.0 absorbance unit.

UV Standard Marker Compounds. Authentic cytosine, 5-methylcytosine, thymine, and 2′-deoxy-5-methylcytidine were purchased from Sigma.

Synthesis of Radioactive Alternating DNA Copolymers. Poly(dG−[^{3}H]dmC) was synthesized from poly(dG−dC) by incubation with CpG methylase and [^{3}H]-*S*-adenosyl-L-methionine. Each reaction contained 250 μCi of [^{3}H]-*S*-adenosyl-L-methionine and 60 μg of poly(dG−dC). Reactions were incubated overnight at 37 °C. The DNA was separated from unincorporated radioactive material by ethanol precipitation, followed by passage through a Sephadex G-50 minicolumn. The purified DNA copolymer was dissolved in 40 mM potassium phosphate and 1 mM EDTA buffer (pH 7.4).

The formation of [^{3}H]-5-methylcytosine within the polymer was confirmed by enzymatically digesting a sample of copolymer and measuring the yield of [^{3}H]-5-methylcytosine as the 2′-deoxyribonucleoside by HPLC analysis. The poly(dG−[^{3}H]dmC) solution was made 0.015 M with respect to $MgCl_2$, followed by treatment at 37 °C with DNase I, alkaline phosphatase, spleen and snake venom phosphodiesterases, and then alkaline phosphatase again overnight. The hydrolysates were precipitated with 5 vol of acetone and centrifuged. Supernatants were evaporated, and the residues were dissolved in water, mixed with authentic 2′-deoxy-5-methylcytidine as a UV marker, and analyzed by HPLC (*vide supra*). A total of 99.6% of recovered radioactivity eluted with the UV marker 2′-deoxy-5-methylcytidine. The specific activity of the copolymer was typically (1.5−2) × 10^6 dpm/μg, indicating that about 40% of the cytosine residues in the copolymer were methylated.

Poly(dG−[^{3}H]dC) and poly(dA−[^{3}H]dT) were made from poly(dG−dC) and poly(dA−dT), respectively, by nick-translation. Each translation reaction was conducted using 10 μCi of [^{3}H]dCTP or [^{3}H]dTTP and 0.5 μg of poly(dG−dC) or poly(dA−dT) in the presence of *E. coli* DNA polymerase I and DNase I. Reaction mixtures were incubated for 2 h at 15 °C and stopped by the addition of 5 μL of 0.25 M EDTA, followed by heating for 15 min at 70 °C. Nick-translated DNA was separated from unincorporated radioactive nucleotide by using a Sephadex G-50 column, followed by ethanol precipitation. The precipitated, purified DNA copolymer was resuspended in 40 mM potassium phosphate and 1 mM EDTA buffer (pH 7.4).

UV Irradiation. Poly(dG−[^{3}H]dmC), poly(dG−[^{3}H]dC), and poly(dA−[^{3}H]dT) were irradiated with two General Electric 15 W germicidal bulbs with a primary output at 254 nm. The incident dose rate was measured with a UVX 254 radiometer (UVP Inc., San Gabriel, CA). Irradiation of DNA was carried out in weighing boats on ice to minimize evaporation. After UV irradiation, each sample was precipitated by the addition of 20 μL of BSA (500 μg) and 500 μL of cold acetone. Acetone-insoluble material was separated by centrifugation, and the acetone-soluble material was dried by evaporation under nitrogen, resuspended in 200 μL of water, and analyzed for the presence of ^{3}H-containing material by HPLC. This fraction represents the nonenzymatically mediated released material. UV irradiation of [*methyl*-^{3}H]thymine was performed at 0.15 mM in the same buffer as the polynucleotides. An aliquot of the irradiated solution was analyzed by HPLC for comparison with the profile of soluble fractions of the UV-irradiated polynucleotides.

Enzymatic Assay of ^{3}H-Containing Photoproducts in Irradiated ^{3}H-Containing Copolymers. Prior to enzymatic assay, UV-irradiated ^{3}H-labeled copolymers were passed through a Sephadex G-50 column to remove any low molecular weight material released from the copolymer as a consequence of the UV irradiation. One microgram of such purified copolymer was incubated with 1 μg of endonuclease III in a final volume of 100 μL containing 40 mM potassium phosphate (pH 7.4) and 1 mM EDTA for 2 h at 37 °C. The reaction was stopped by the addition of 20 μL of BSA (500 μg) and 500 μL of cold acetone. Acetone-insoluble material was separated by centrifugation, and the acetone-soluble material was dried by evaporation under nitrogen, resuspended in 200 μL of water, and analyzed for the presence of ^{3}H-containing material by HPLC. This fraction represents the enzymatically repairable photoproducts.

HPLC Analysis and Quantitation of ^{3}H-Containing Material Released from Copolymers either by UV Irradiation Alone or after Incubation with Endonuclease III. Samples were analyzed on a 5 μm, 0.46 × 25 cm Ultrasphere ODS column (Beckman) using 50 mM ammonium formate and 0.5% methanol (pH 6.8) as the isocratic eluant at a rate of 1 mL/min. The elution of radioactive material was monitored via a Radiomatic Flo-One on-line radioactivity detector. All quantitative data reported in these experiments are derived from samples separated by HPLC and monitored for radioactive content by the on-line radioactivity detector. All data reported in the figures and the table are calculated from the total radioactivity eluted from the HPLC. UV marker compounds cytosine, thymine, and 5-methylcytosine were added to the samples, and the elution of these internal standards was monitored by on-line UV absorbance.

Thin-Layer Chromatography (TLC). Samples of UV-released material were initially separated by HPLC and further analyzed by TLC. If silica gel plates were used as the solid phase, the chromatographs were developed using the lower phase of a chloroform/methanol/water (4:2:1) mixture to which 5 mL of methanol had been added for each 100 mL of organic phase (Teoule et al., 1974; Cadet et al., 1976; Boorstein et al., 1989). If PEI papers were used as the solid phase, the chromatographs were developed with 0.15 M LiCl solution. Following chromatography, regions coeluting with the UV fluorescent markers were outlined, scraped into scintillation vials, and analyzed by liquid scintillation counting.

Chemical Synthesis of cis-Thymine Hydrate. Thymine (125 mg) and 125 μL of [^{14}C]thymine (12.5 μCi) were suspended in 1.5 mL of bromine-saturated water and stirred for 60 min. Acetic acid (75 μL) and 250 mg of zinc powder were added and kept shaking for 60 min. The reaction

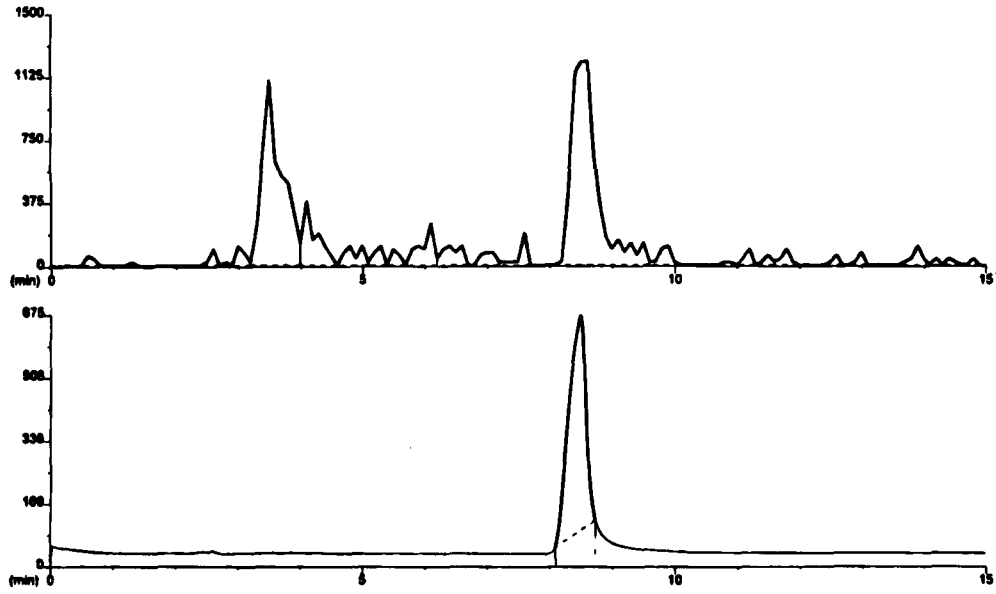

FIGURE 1: HPLC profile of acetone-soluble products released from poly(dG−[^{3}H]dmC) by UV irradiation. The upper panel shows the radioactivity released by UV irradiation. The lower panel shows the A_{254} of the eluant to which 5-methylcytosine was added as a nonradioactive UV marker. The ordinate of the upper panel is dpm. The ordinate of the UV panel is an arbitrary sensitivity scale. The abscissa is time in minutes.

mixture was filtered, lyophilized, and HPLC purified, yielding *cis*-thymine hydrate (Cadet & Teoule, 1971).

UV Irradiation in the Presence or Absence of Oxygen. UV irradiation in the presence of oxygen (room air) was carried out according to the methods described previously. UV irradiation in the absence of oxygen was carried out by irradiating the DNA polymers in a specially designed quartz NMR tube (Wilmad, Buena, NJ), through which argon was bubbled to eliminate oxygen. Control samples were irradiated without argon bubbling. The products of nonenzymatically or enzymatically mediated release from the UV-irradiated copolymers were analyzed by HPLC.

UV Irradiation in the Presence of Free Radical Scavengers and an Iron Chelator. Scavengers Thiourea, DMSO, and Mannitol and a chelator desferal (deferoxamine mesylate, CIBA) were added separately to the DNA samples prior to UV irradiation. UV irradiation and HPLC analysis were performed as described.

RESULTS

Characterization of the ^{3}H-Containing Material Released from UV-Irradiated Poly(dG−[^{3}H]dmC). Poly(dG−[^{3}H]-dmC) was irradiated with 0−400 kJ m^{-2} of 254 nm UV light. After precipitation, the acetone-soluble material was dried, redissolved in water, and analyzed by HPLC.

A representative HPLC profile is shown in Figure 1. All radioactive material was derived from the [^{3}H]-5-methylcytosine residues within the copolymer. The early eluting material was heterogeneous, but a single peak coeluted with the authentic 5-methylcytosine UV marker. The chemical identity of the ^{3}H-containing peak that coeluted with 5-methylcytosine was confirmed by TLC. First, a sample was separated by HPLC. The HPLC fractions containing the ^{3}H-containing products were dried, redissolved in methanol, and spotted onto 10 × 10 cm silica gel TLC plates. In this chromatographic system, 5-methylcytosine had an R_f value of 0.76. The ^{3}H-containing material that had coeluted with authentic 5-methylcytosine in HPLC comigrated with authentic 5-methylcytosine on TLC.

To determine whether the material eluting before 5-methylcytosine contained oligonucleotides formed as a consequence of strand breakage during irradiation, analysis by TLC using PEI anion exchange paper as the solid phase and 0.15 M LiCl solution as the mobile phase was performed. Oligonucleotides or mononucleotides, which bear negatively charged phosphate groups, bind firmly to the PEI paper and remain at the origin in this system. The early eluting radioactive material moved with the solvent front, indicating that it did not contain charged phosphate groups and therefore was neither a mono- nor an oligonucleotide.

When the early eluting material was heated in 0.1 N HCl at 90 °C for 2 h, neither 5-methylcytosine nor thymine appeared. There was also no change in the general shape of the early eluting material and no new peak appeared. Thus, the stability to both heat and acid precluded there being any 5-methylcytosine hydrate or thymine hydrate within the complex mixture since, under the conditions employed, they would eliminate water, yielding the parent compounds (Cadet & Teoule, 1971).

After irradiation of poly(dG−[^{3}H]dmC) with the maximum 400 kJ m^{-2}, about 0.5% of the 5-methylcytosine residues in the copolymer were released by UV irradiation alone in the absence of repair enzyme.

Assay for the Presence of Enzymatically Repairable Products Using Endonuclease III. To determine whether UV irradiation also caused the formation of stable endonuclease III-repairable products within the DNA, UV-irradiated poly(dG−[^{3}H]dmC) was first passed through a Sephadex G-50 column to remove the UV-induced nonenzymatically released low molecular weight material shown in Figure 1 and then incubated with 1 μg of endonuclease III for 2 h at 37 °C. No additional radioactive material was released from the copolymer (Figure 2). The small amount of radioactive material eluting at the retention time of 5-methylcytosine was seen with or without the addition of endonuclease III and probably represents the slow release of labilized 5-methylcytosine. These results lead us to conclude that neither 5-methylcytosine hydrate nor thymine hydrate was formed

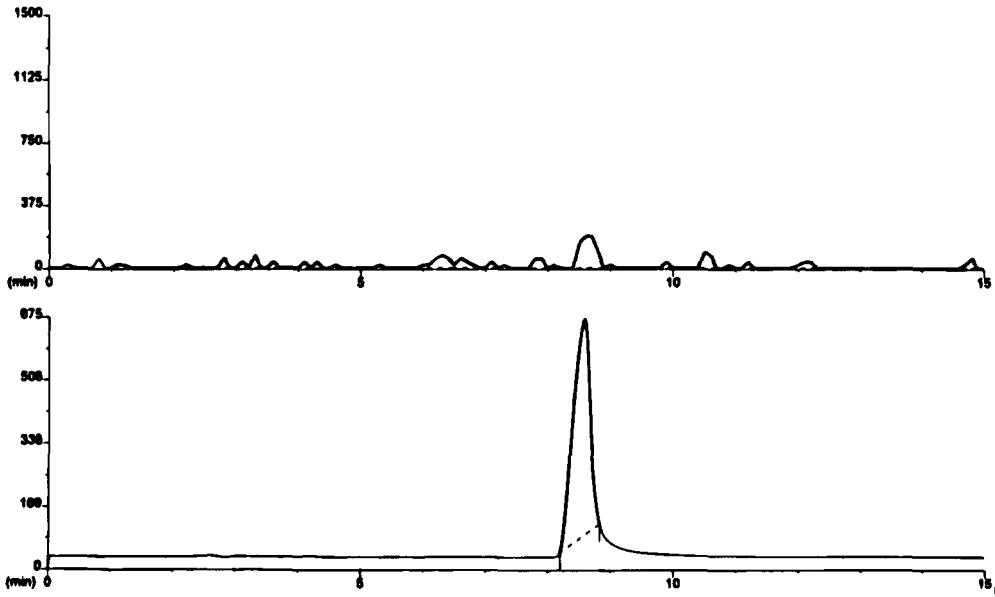

FIGURE 2: HPLC profile of acetone-soluble products released from UV-irradiated poly(dG−[^{3}H]dmC) by endonuclease III. The upper panel shows the radioactivity released by UV irradiation. The lower panel shows the A_{254} of the eluant to which 5-methylcytosine was added as a nonradioactive UV marker. The ordinate of the upper panel is dpm. The ordinate of the UV panel is an arbitrary sensitivity scale. The abscissa is time in minutes.

to any significant degree during UV irradiation of the 5-methylcytosine-containing copolymer, since pyrimidine photohydrates within DNA are substrates for endonuclease III.

Determination of How the 5-Methyl Group Affects the Sensitivity to UV Radiation of 5-Methylcytosine and Thymine in an Alternating DNA Copolymer Compared to Cytosine in an Alternating DNA Copolymer. UV irradiation of the 5-methylcytosine-containing copolymer resulted in the nonenzymatically mediated release of 5-methylcytosine but no release of pyrimidine hydrates by endonuclease III. This result was in striking contrast to our previous results with poly(dG−[^{3}H]dC), where identical doses of radiation resulted in exactly the opposite result, e.g., a very small amount of nonenzymatically mediated release of cytosine and UV dose-dependent formation of endonuclease III-repairable cytosine hydrate and its deamination product uracil hydrate (Boorstein et al., 1989, 1990).

Ganguly et al. (1990), using poly(dA−[^{3}H]dT), reported results similar to those we obtained with poly(dG−[^{3}H]dC). They synthesized [^{14}C]thymine hydrate and reported the release of ^{3}H-containing material that coeluted with their ^{14}C marker compound. In light of our failure to detect any endonuclease III-sensitive material in irradiated poly(dG−[^{3}H]dmC), we irradiated poly(dA−[^{3}H]dT) to determine whether we could duplicate the results of Ganguly et al. and simultaneously irradiated poly(dG−[^{3}H]dC) as a positive control for our methodology.

Nick-translated poly(dA−[^{3}H]dT) and poly(dG−[^{3}H]dC) were UV irradiated for 0−400 kJ m^{-2} under the same conditions of irradiation that were used for alternating poly(dG−[^{3}H]dmC) and were then incubated with or without endonuclease III. The released photoproducts were analyzed by HPLC. The profile of Figure 3 shows the material that was nonenzymatically released from the UV-irradiated poly(dA−[^{3}H]dT). The overall pattern was similar to that of Figure 1, containing both heterogeneous early eluting thymine-derived material and a peak that coeluted with authentic marker thymine. The identity of the thymine was confirmed by TLC just as had been done for 5-methylcytosine.

To determine whether thymine hydrate was formed in DNA, the UV-irradiated poly(dA−[^{3}H]dT) was purified by passage through a Sephadex G-50 column to remove low molecular weight material released as a consequence of the UV irradiation. When the purified copolymer was incubated with endonuclease III at 37 °C for 2 h, no additional material was released except for the small amount of material coeluting with thymine, which is independent of the presence of the enzyme (Figure 4). This figure shows data from three channels. The upper channel is the ^{3}H channel demonstrating the absence of release of any material by endonuclease III. The middle channel is the ^{14}C channel demonstrating the authentic thymine hydrate molecule that we synthesized with a retention time of 7.6 min (*vide infra*) and authentic thymine with a retention time of 14 min. The bottom channel is the UV absorbance channel with authentic thymine. Incubation with partially purified mammalian 5-hydroxymethyluracil DNA glycosylase also failed to release any material.

Thus, our results indicate that, under the conditions of UV irradiation employed, no thymine hydrates were formed in poly(dA−[^{3}H]dT), nor was there formation of any other modified pyrimidines repairable by either endonuclease III or 5-hydroxymethyluracil DNA glycosylase. The total amount of UV-induced damage by the 400 kJ m^{-2} of radiation was about 0.8% of the thymine residues in DNA, all of which were released by UV radiation from the DNA backbone. These results contrasted with those obtained by UV irradiation of poly(dG−[^{3}H]dC), in which identical doses of UV radiation caused only 0.2% of cytosine residues to be nonenzymatically released from the DNA backbone (Figure 5). Incubation of UV-irradiated poly(dG−[^{3}H]dC) with endonuclease III resulted in the release of cytosine hydrate (1.9%), just as we previously described (Boorstein et al., 1989, 1990) (Figure 6).

Further Confirmation That Thymine Hydrates Were Not Formed in UV-Irradiated DNA. To further confirm that

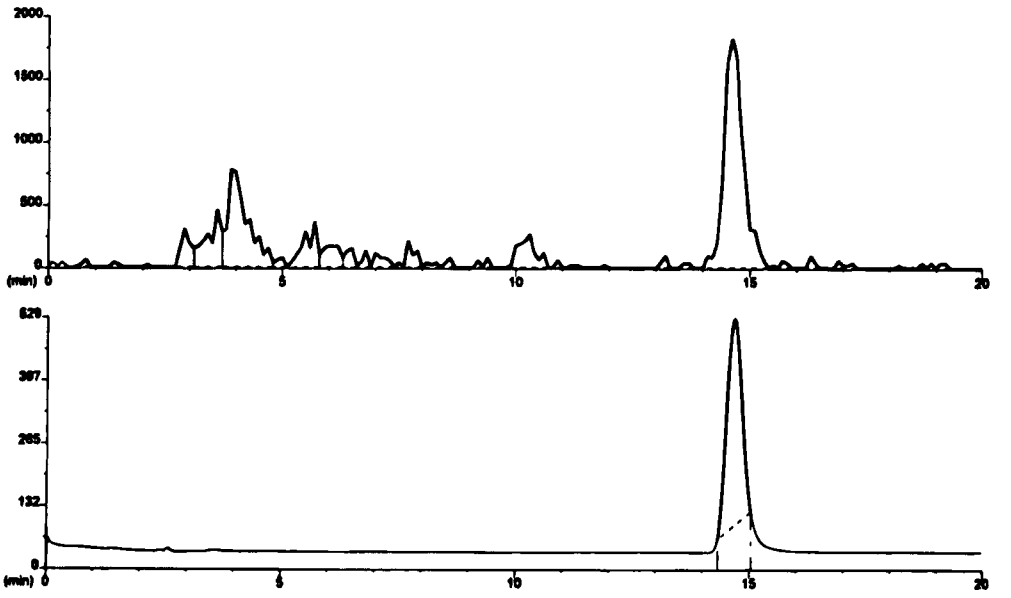

FIGURE 3: HPLC profile of acetone-soluble products released from poly(dA−[^{3}H]dT) by UV irradiation. The upper panel shows the radioactivity released by UV irradiation. The lower panel shows the A_{254} of the eluant to which thymine was added as a nonradioactive UV marker. The ordinate of the upper panel is dpm. The ordinate of the UV panel is an arbitrary sensitivity scale. The abscissa is time in minutes.

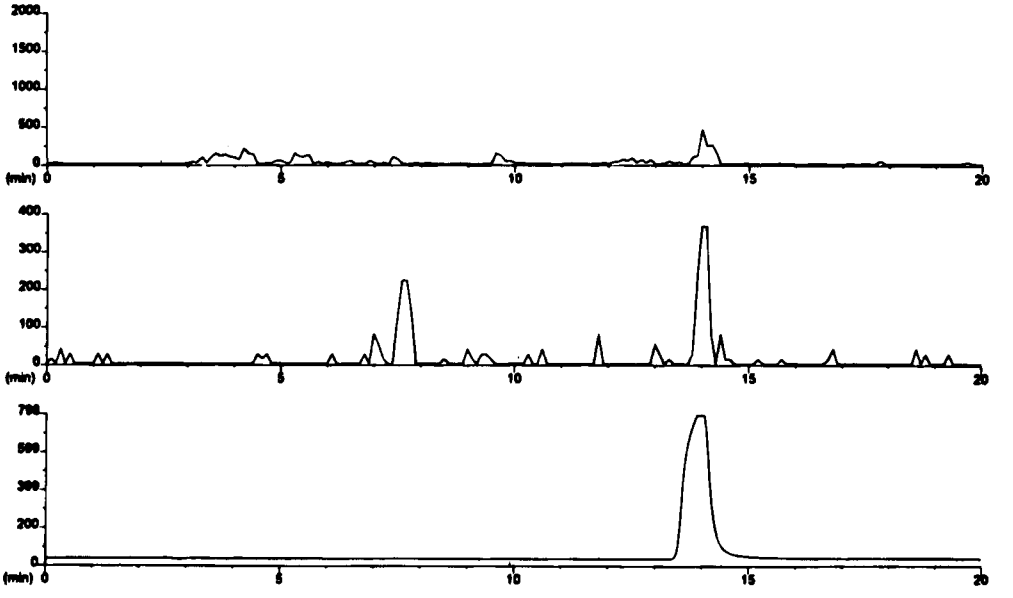

FIGURE 4: HPLC profile of acetone-soluble products released from UV-irradiated poly(dA−[^{3}H]dT) by endonuclease III. The upper panel shows the radioactivity released by UV irradiation. The middle panel shows [^{14}C]thymine and the authentic chemically synthesized [^{14}C]-thymine hydrate. The lowest panel shows the A_{254} of the eluant to which thymine was added as a nonradioactive UV marker. The ordinate of the upper panels is dpm. The ordinate of the UV panel is an arbitrary sensitivity scale. The abscissa is time in minutes.

thymine hydrates were not formed in DNA during UV irradiation, nor were among the nonenzymatically released products, the chemical synthesis of *cis*-[^{14}C]thymine hydrate as a radioactive marker compound was carried out as described in Materials and Methods. The *cis*-thymine hydrate so synthesized completely reverted to thymine upon heating in 0.1 N HCl for 15 min at 90 °C, proving that we had indeed synthesized authentic *cis*-thymine hydrate (data not shown). Thymine hydrate had a retention time of 7.6 min when eluted with 50 mM ammonium formate (pH 6.8) and 0.5% methanol from an ODS column, which was the same solvent we used in the HPLC analysis of the supernatant material recovered from the solutions of irradiated copolymers after acetone precipitation. None of the nonenzymatically mediated released material coeluted with marker compound thymine hydrate, nor, as stated earlier, was any such compound released from irradiated poly(dA−[^{3}H]dT) by endonuclease III.

Irradiation of Poly(dA−[^{3}H]dT), Poly(dG−[^{3}H]dmC), and Poly(dG−[^{3}H]dC) in the Absence of Oxygen. The UV irradiation-induced release of thymine, 5-methylcytosine, and cytosine residues from the copolymer backbone was evidence that a reaction other than the addition of water to the 5,6-double bond of the pyrimidines had taken place. In addition to water, another potentially reactive molecule present within the reaction mixture was oxygen. To determine whether the release of pyrimidines was dependent on oxygen, we irradiated poly(dA−[^{3}H]dT), poly(dG−[^{3}H]dmC), and poly(dG−[^{3}H]dC) under argon (L. Loeb, personal communication). This condition resulted in 72% inhibition of base

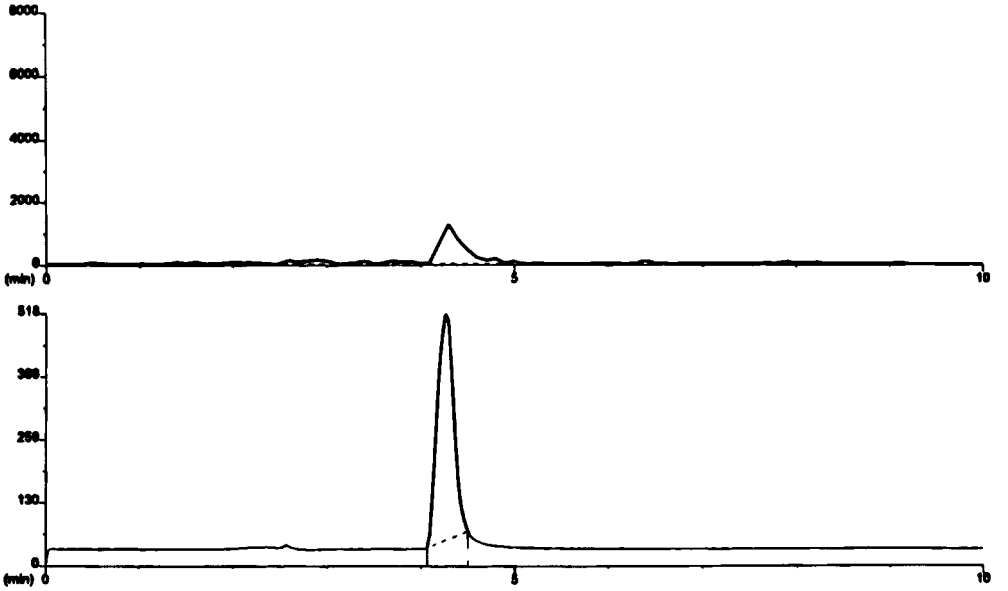

FIGURE 5: HPLC profile of acetone-soluble products released from poly(dG−[^{3}H]dC) by UV irradiation. The upper panel shows the radioactivity released by UV irradiation. The lower panel shows the A_{254} of the eluant to which cytosine was added as a nonradioactive UV marker. The ordinate of the upper panel is dpm. The ordinate of the UV panel is an arbitrary sensitivity scale. The abscissa is time in minutes.

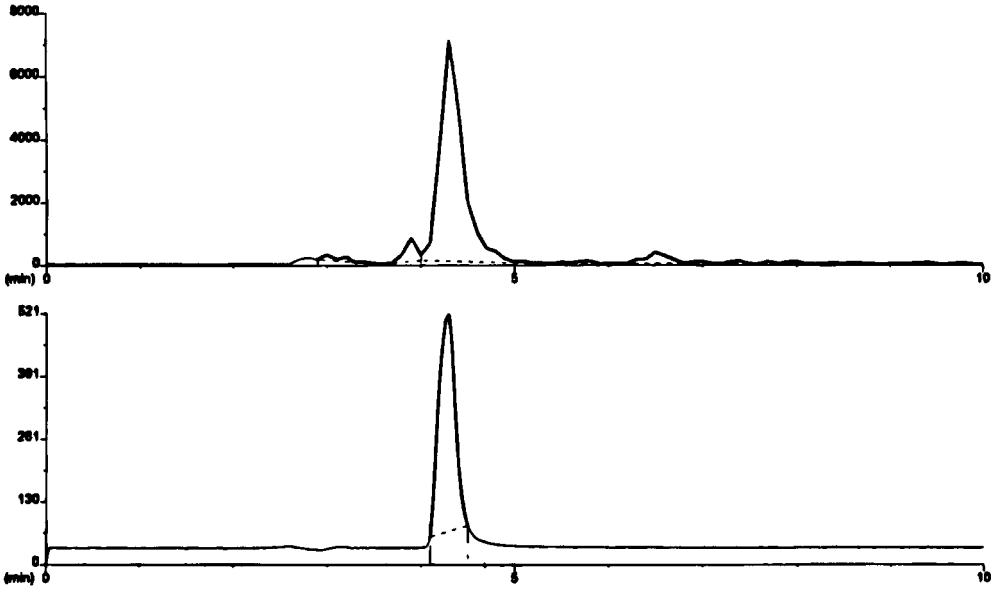

FIGURE 6: HPLC profile of acetone-soluble products released from UV-irradiated poly(dG−[^{3}H]dC) by endonuclease III. The upper panel shows the radioactivity released by UV irradiation. The lower panel shows the A_{254} of the eluant to which cytosine was added as a nonradioactive UV marker. The ordinate of the upper panel is dpm. The ordinate of the UV panel is an arbitrary sensitivity scale. The abscissa is time in minutes.

release from poly(dA−[^{3}H]dT), 57% inhibition of base release from poly(dG−[^{3}H]dmC), and 91% inhibition of base release from poly(dG−[^{3}H]dC). When poly(dA−[^{3}H]dT) or poly(dG−[^{3}H]dmC), which had been irradiated under argon, was incubated with endonuclease III, no repairable photohydrates were released. In sharp contrast, when poly(dG−[^{3}H]dC) was UV irradiated under argon, the yield of enzymatically released cytosine hydrate was unaffected by the absence of oxygen (data not shown). Thus, these experiments demonstrated that the UV-induced release of thymine-, 5-methylcytosine-, or cytosine-derived materials was oxygen dependent, while the formation of cytosine photohydrates within DNA was oxygen independent.

UV Irradiation in the Presence of Free Radical Scavengers and an Iron Chelator. To determine whether free radical species were formed during irradiation, and whether they played a role in nonenzymatically mediated base release, free radical scavengers (DMSO, mannitol, and thiourea) were added to the reaction mixture. The results are summarized in Table 1. When poly(dA−[^{3}H]dT), which had been irradiated in the presence of thiourea, was subsequently incubated with endonuclease III, no release of radioactive material was observed, indicating that no enzymatically repairable thymine hydrates were formed via some alternate reaction.

The preceding results strongly suggested that a free radical intermediate was formed during the irradiation. Since iron is the most common transition metal involved in such reactions, and adventitial iron is present in most organic salts, we added an iron chelator to the solution to determine

Table 1: Amounts of Pyrimidine Residues in Poly(dA−[^{3}H]dT), Poly(dG−[^{3}H]dmC), and Poly(dG−[^{3}H]dC) Copolymers Released by 400 kJ m^{-2} of UV (254 nm) Irradiation under Specified Conditions

	poly(dA−[^{3}H]dT)		poly(dG−[^{3}H]dmC)		poly(dG−[^{3}H]dC)	
conditions of irradiation	pmol/μg	% inhibition	pmol/μg	% inhibition	pmol/μg	% inhibition
room air	11.0		8.7		3.5	
argon	3.1	72	3.7	57	0.3	91
desferal (5 mM)	3.3	70	2.9	67	0.3	91
DMSO (10%)	7.9	28	3.0	66	0.3	91
mannitol (100 mM)	7.2	35	5.1	41	0.7	80
thiourea (5 mM)	1.4	87	1.5	83	0.1	97

whether inhibition of base release occurred. The addition of desferal (*vide supra*) to a final concentration of 5 mM resulted in 70% inhibition of base release from poly(dA−[^{3}H]dT), 67% inhibition of base release from poly(dG−[^{3}H]-dmC), and 91% inhibition of base release from poly(dG−[^{3}H]dC). This result strongly suggested that trace amounts of iron had indeed participated in the reaction. Therefore, we asked whether the addition of iron would enhance the response.

The addition of 5 μM $FeCl_3$ to the DNA samples prior to UV irradiation at 100 kJ m^{-2} increased the amount of base release by 6-fold for poly(dA−[^{3}H]dT), 3-fold for poly(dG−[^{3}H]dmC), and 3-fold for poly(dG−[^{3}H]dC). These results are summarized in Figure 7A,B, in which the amount of material released in the absence or presence of added ferric ion is plotted as a function of incident UV dose. The addition of the same amount of iron after irradiation did not result in further base release. This negative result indicated that no long-lived peroxide-like molecules persisted to any significant degree after the irradiation was terminated. The HPLC profiles of the nonenzymatically mediated released products were qualitatively similar to those of the control samples, to which no exogenous iron salt had been added, indicating that there was no change in the chemistry of the reaction. The addition of Fe^{3+} did not affect photohydrate formation from cytosine.

DISCUSSION

These experiments yielded positive and negative results, which may be explained from the standpoint of DNA photochemistry. The negative result was our inability to reproduce previously published data demonstrating the photohydration of 5-methylpyrimidines in DNA. Our experiments were done under conditions identical in detail to those described by Ganguly and Duker, except for the light source. Our source was the commonly used low-pressure germicidal bulb, in contrast to their 200 W high-pressure mercury lamp of "mixed wavelength". Their dose rate was described as "12 J/m^2/sec for the 270 nm component" (Ganguly & Duker, 1991), which may be compared to our rate of 56 J/m^2/s measured with a 254 nm sensor. Under these conditions, we irradiated for 2 h and they for only 1 h. Thus, as a best estimate, the total irradiation doses seem to have been similar.

Additionally, we chemically synthesized *cis*-thymine hydrate and demonstrated its quantitative reversion to thymine upon acidification and heating. No such compound was spontaneously or enzymatically released from the irradiated 5-methylpyrimidine-containing copolymers. Thus, we conclude that 5-methylpyrimidines in DNA do not undergo photohydration to a meaningful degree compared to 5-unsubstituted cytosine.

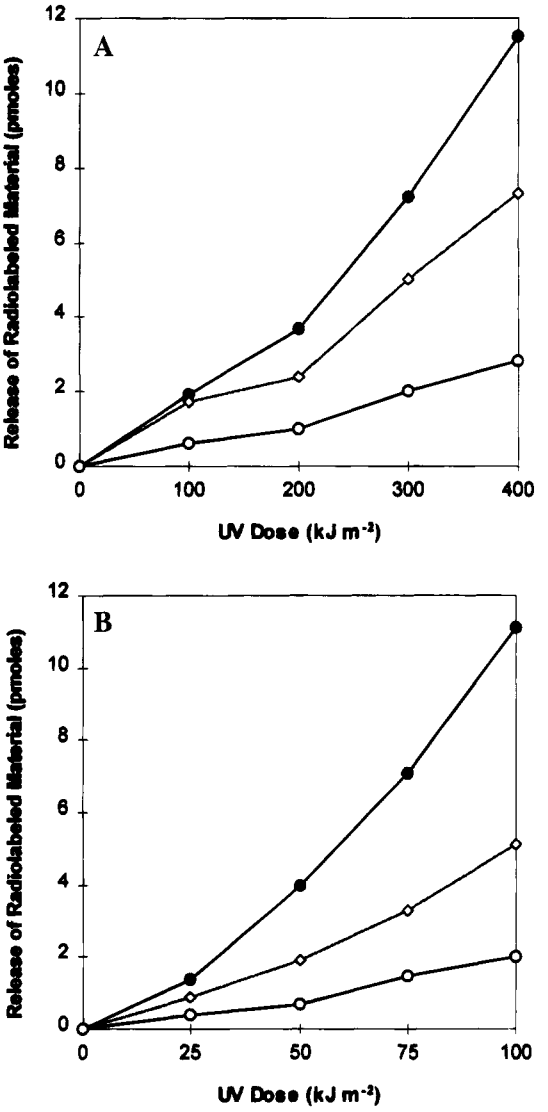

FIGURE 7: (A) Plot of the amount of radiolabeled material released nonenzymatically by UV irradiation in the absence of ferric irons vs UV dose from poly(dA−[^{3}H]dT) (●), poly(dG−[^{3}H]dmC) (◇), and poly(dG−[^{3}H]dC) (○). (B) Plot of the amount of radiolabeled material released nonenzymatically by UV irradiation in the presence of 5 μM ferric irons vs UV dose from poly(dA−[^{3}H]dT) (●), poly(dG−[^{3}H]dmC) (◇), and poly(dG−[^{3}H]dC) (○).

The positive result was the UV-induced nonenzymatically mediated base release that we observed. There is venerable precedent for this observation. Alexander and Moronson (1960) demonstrated a decrease in the intrinsic viscosity of a DNA solution irradiated with 254 nm light, implying that strand breakage had occurred. This decrease was dependent

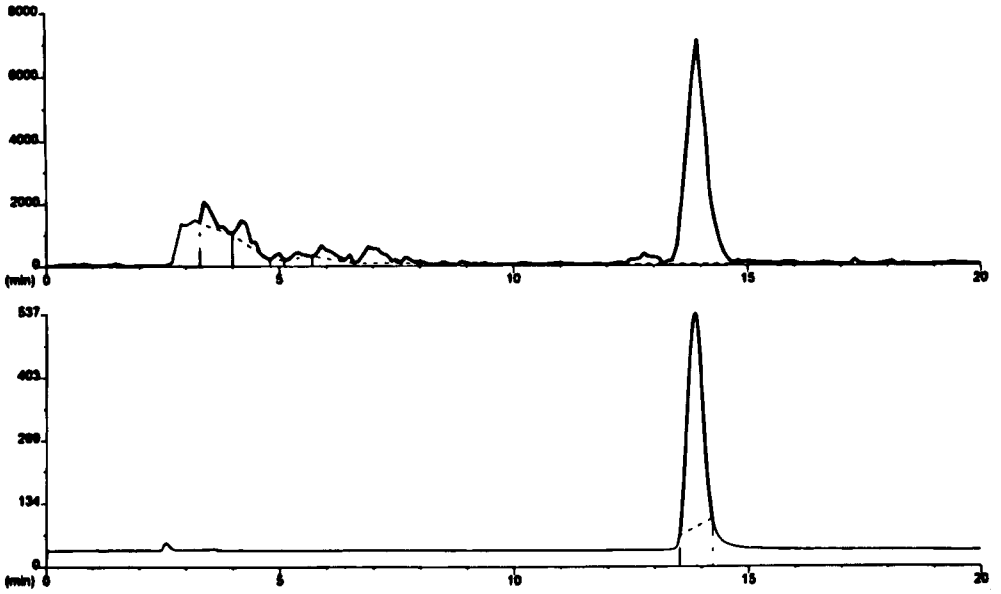

FIGURE 8: HPLC profile of UV-irradiated [*methyl*-^{3}H]thymine. The upper panel shows the ^{3}H radioactivity. The lower panel shows the A_{254} of the eluant to which thymine was added as a nonradioactive UV marker. The ordinate of the upper panel is dpm. The ordinate of the UV panel is an arbitrary sensitivity scale. The abscissa is time in minutes.

upon the presence of oxygen. When irradiation was done under nitrogen, the decrease in viscosity was significantly reduced.

More recently, Audic and Giacomoni (1993) demonstrated enhancement of UV-induced nicking of plasmid DNA in the presence of added iron and/or oxygen at 290, 313, and 365 nm. Nicking was maximal at 290 nm and minimal at 365 nm, reflecting the absorption spectrum of DNA in solution. Nicking was reduced by over 2 orders of magnitude after argon was bubbled through the solution for 15 min. Audic and Giacomoni suggested that the reaction they observed might be mediated by UV-induced electron transfer from DNA to iron, resulting in the formation of an AP site and ultimate chain breakage. A similar result was observed by Larson et al. (1992), who irradiated DNA with UVA light in the presence of ferric ion varying in concentration from 0 to 30 μM and demonstrated an oxygen dependent reduction in transforming activity in bacterial systems as a function of the iron concentration.

Our results indicate that iron played a role in the UV-induced release of the 5-methylpyrimidine-derived material that we observed. In the absence of exogenous iron, the addition of desferal, an iron chelator, inhibited the reaction by chelating endogenous iron, while the addition of iron greatly enhanced the reaction.

The literature is replete with data suggesting that activated oxygen plays a role in UV toxicity (Kondo & Nishioka, 1991; Hanada et al., 1993; Dudek et al., 1993). Two possible reactions of triplet-excited pyrimidine bases with oxygen have been proposed (Bishop et al., 1994). One involves energy transfer from the UV-excited nucleobase to ground state oxygen, resulting in the formation of singlet oxygen. The other involves electron transfer from the UV-excited nucleobases to oxygen, leading to the formation of superoxide and its derivatives, such as hydrogen peroxide and hydroxyl radicals.

Singlet oxygen probably does not account for the results seen during UV irradiation of the copolymers, since the substitution of D_2O for H_2O did not result in an increase in the net amount of base release (data not shown). The amplification of the UV-induced effect by the addition of ferric iron and the partial inhibition of the effect by free radical scavengers may be taken as evidence for the involvement of free radicals in the reaction. The transfer of an electron from the UV-excited triplet of thymine or 5-methylcytosine to the ground state oxygen triplet could yield reactive oxygen species (Kemp et al., 1985, 1987; Bishop et al., 1994), such as superoxide or hydrogen peroxide, and in the presence of iron, the hydroperoxide could yield free radicals or other oxidative intermediates (Wink et al., 1994), which could attack the *N*-glycosyl bonds in DNA. Alternatively, as suggested by Audic and Giacomoni (1993) and Larson et al. (1992), electron transfer may go first to the ferric ion followed by interaction with molecular oxygen.

Whatever the primary electron acceptor, it is not surprising that this type of photochemically induced oxygen dependent damage was primarily seen with the substituted 5-methylpyrimidines, since the quantum yield of the UV-induced excited triplet in thymine is 1 order of magnitude greater than that of UV-irradiated unsubstituted cytosine (Fisher & Johns, 1976b). In contrast, the failure of the excited triplet state 5-methylpyrimidines to undergo photohydration is also consistent with evidence indicating that photohydrates arise from the excited singlet state characteristic of 5-unsubstituted cytosine (Cadet & Vigny, 1990).

The actual nature of the reactive radical species formed by this reaction is not certain, but the partial protection afforded by free radical scavengers indicates that OH radicals are not responsible for the observed effects. Similar results were seen by Larsen et al. (1992), who also concluded that the radical species generated by UVA light in the presence of DNA, oxygen, and iron were not OH radicals. Although the nonenzymatically mediated release of intact pyrimidines from DNA that we observed is also characteristic of the *in vitro* effects of ionizing radiation on DNA in solution (Von, 1987), the characteristic OH radical-mediated oxidation of the base moieties, notably thymine, yielding thymine glycol

and 5-hydroxymethyluracil, was not observed in these experiments.

The chemical nature of the early eluting peaks of the HPLC profiles of Figures 1 and 3 was not unambiguously determined due to their heterogeneity. We suggest that these are dimeric photoproducts formed from the undamaged 5-methylpyrimidine bases after their release from the copolymer backbone. To test this hypothesis, we irradiated free [^{3}H]thymine and analyzed an aliquot of the solution. A representative HPLC profile of such irradiated thymine is shown in Figure 8. When compared with Figure 3, which shows the radiolabeled material released from poly(dA−[^{3}H]-dT) after UV irradiation, the overall patterns are virtually identical. The retention times of these early eluting peaks are comparable to those reported by Cadet and Voituriez (1980) to represent dimeric photoproducts. In the case of 5-methylcytosine, we known that these compounds are not the UV-induced 3-ureidoacrylonitriles derived from 5-methylcytosine described by Shaw and Shetlar (1990), because they did not coelute with the authentic markers provided to us by Dr. Shetlar (data not shown). The HPLC profile of the material released from poly(dG−[^{3}H]dC) (Figure 5) shows that there was much less of the early eluting material compared to the 5-methylpyrimidines, the major peak being intact cytosine. This is consistent with the findings that the yield of the UV-induced triplet state of thymine is at least 10 times greater than that of cytosine and that the concomitant formation of cytosine dimers is proportionately less (Douki & Cadet, 1994).

Finally, what is the biological significance of such damage? We suggest that the UV sensitivity of *E. coli xthnfo* mutants (Cunningham et al., 1986) can be explained by our data. Those *E. coli* mutants lack AP endonuclease activity, and the type of UV damage we describe here causes the formation of AP sites. Thus, UV irradiation of bacterial cells may yield AP sites in sufficient number, such that repair-deficient cells would show decreased survival. Whether such damage contributes to the overall toxicity and mutagenicity of UV radiation to mammalian species warrants further study.

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