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Ultraviolet-Induced Thymine Hydrates in DNA Are Excised by Bacterial and Human DNA Glycosylase Activities†

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ABSTRACT: Ultraviolet irradiation of DNA results in various pyrimidine modifications. We studied the excision of an ultraviolet thymine photoproduct by *Escherichia coli* endonuclease III and by a preparation of human WI-38 cells. These enzymes cleave UV-irradiated DNA at apyrimidinic sites formed by glycosylic removal of the photoproduct. Poly(dA-[^{3}H]dT)·poly(dA-[^{3}H]dT) was UV irradiated and incubated with purified *E. coli* endonuclease III. ^{3}H-Containing material was released in a manner consistent with Michaelis-Menten kinetics. This ^{3}H-labeled material was determined to be a mixture of thymine hydrates (6-hydroxy-5,6-dihydrothymine), separable from unmodified thymine by chromatography in three independent systems. Both *cis*-thymine hydrate and *trans*-thymine hydrate were chemically and photochemically synthesized. These coeluted with the enzyme-released ^{3}H-containing material. No thymine glycol was released from the UV-irradiated polymer. Similar results were obtained with extracts of WI-38 cells as the enzyme source. The release of thymine hydrates by both glycosylase activities was directly proportional to the amount of enzyme and the irradiation dose to the DNA substrate. These results demonstrate the modified thymine residues recognized and excised by endonuclease III and the human enzyme to be a mixture of *cis*-thymine hydrate and *trans*-thymine hydrate. The repairability of these thymine hydrates suggests that they are stable in DNA and therefore potentially genotoxic.

Endonuclease III of *Escherichia coli* is involved in the initiation of the repair of various pyrimidine damages in DNA. These include photochemical alterations resulting from UV[1] irradiation (Radman, 1976; Gates & Linn, 1977). Chemical oxidation, X-irradiation, and γ-irradiation of DNA also result in base damages that are substrates for this enzyme (Gates & Linn, 1977; Demple & Linn, 1980, 1982; Katcher & Wallace, 1983; Breimer & Lindahl, 1984, 1985). An analogous activity, present in cultured human cells, was originally found to incise UV-irradiated DNA (Brent, 1972; Duker & Teebor, 1975). This human enzyme, like *E. coli* endonuclease III, recognizes monomeric pyrimidine lesions in UV-irradiated DNA and also base damages in OsO_4-oxidized or X-irradiated DNAs (Brent, 1973, 1976, 1983; Doetsch et al., 1987). Studies using DNA sequencing techniques and chemical analyses of reaction products indicate the two enzymes to have an identical range of modified pyrimidine substrates (Boorstein et al., 1989; Doetsch et al., 1986, 1987, 1988; Higgins et al., 1987; Gallagher et al., 1989a,b; Lee et al., 1988; Weiss et al., 1989). Common structural features of these bases include saturation of the 5,6-double bond or loss of ring planarity (Breimer & Lindahl, 1985; Teoule, 1987; Teebor et al., 1988). It has been recently established that both enzymes remove cytosine hydrate (6-hydroxy-5,6-dihydrocytosine) and uracil hydrate (6-hydroxy-5,6-dihydrouracil) from UV-irradiated DNA (Boorstein et al., 1989; Weiss et al., 1989; Ganguly & Duker, 1990). This type of enzyme, sometimes termed a "redoxyendonuclease", is widely conserved and present in a wide variety of species. These enzymes contain two activities that act sequentially: a DNA glycosylase, which releases the modified pyrimidines, and a DNA apurinic/apyrimidinic site endonuclease, which incises DNA at the resultant apyrimidinic sites by catalysis of β-elimination. They are well discussed in a number of recent reviews (Weiss & Grossman, 1987; Sancar & Sancar, 1988; Wallace, 1988).

The DNA glycosylase activity of endonuclease III releases 5,6-saturated thymine and its derivatives, such as thymine glycol (5,6-dihydroxy-5,6-dihydrothymine), 5-hydroxy-5-methylhydantoin, methyltartronylurea, and urea, as free bases from oxidized DNA (Demple & Linn, 1980; Katcher &

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[1] Abbreviations: UV, ultraviolet; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; assay buffer, 40 mM KH_2PO_4, pH 7.4, and 1 mM EDTA; 6-4 photoproduct, 6-(1,2-dihydro-2-oxo-4-pyrimidinyl)-5-methyl-2,4(1*H*,3*H*)-pyrimidinedione.

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Wallace, 1983; Breimer & Lindahl, 1984, 1985; Higgins et al., 1987). A monohydroxydihydrothymine moiety was also released by this enzyme from DNA that had been γ-irradiated under hypoxic conditions, but the identity of this product was not established (Breimer & Lindahl, 1985). The formation of an uncharacterized thymine photoproduct in a UV-irradiated restriction fragment from pUC18 plasmid and its incision by an activity in Hela cells were reported (Doetsch et al., 1988). To further investigate the mechanism of action of *E. coli* endonuclease III and the human enzyme and to characterize the released thymine UV photoproduct, we used UV-irradiated poly(dA-dT)·poly(dA-dT), labeled in thymine, to demonstrate that both *cis*-thymine hydrate and *trans*-thymine hydrate (6-hydroxy-5,6-dihydrothymine) are formed in DNA, that they are stable, and that both are excised by glycosylic activities of *E. coli* endonuclease III and human cells.

Experimental Procedures

Preparation and Ultraviolet Irradiation of Radiolabeled Substrates. The double-stranded polydeoxyribonucleotide poly(dA-dT)·poly(dA-dT) was radiolabeled at thymine with [5-^{3}H]TTP (NEN, NET-221H; 20.0 Ci/mmol) by nick translation (Maniatis et al., 1982). It was dissolved in 40 mM KH_2PO_4, pH 7.4, containing 1 mM EDTA (assay buffer). The specific activity of the substrate was approximately 2.5 × 10^6 cpm/μg. Mixed-wavelength irradiation was with a Bausch & Lomb unit equipped with a 200-W Osram Hg bulb and a Schott WG 320 filter at an incident dose of 6.6 × 10^{-5} einstein cm^{-2} min^{-1} as previously described (Duker et al., 1982, 1985).

Enzyme Preparations. Endonuclease III was purified from overproducing *E. coli* strain λ-N99 containing the plasmid pHIT1 by the method of Weiss and Duker (1986). Human WI-38 cells were grown and prepared for assay as previously described (Duker & Teebor, 1975; Ganguly & Duker, 1990). Protein contents were measured with the Bio-Rad protein assay kit.

Assays for DNA Glycosylase Activities. The enzymic release of the radiolabeled thymine photoproduct into an ethanol-soluble fraction was determined by use of UV-irradiated poly(dA-dT)·poly(dA-dT); 30 and 60 ng of UV-irradiated substrate were incubated at 37 °C with either endonuclease III or the human cell extract in a reaction volume of 100 μL of assay buffer. The reaction was stopped by placing the reaction mixtures in ice, followed by addition of 100 μL of calf thymus DNA (1 mg/mL), 30 μL of 0.3 M sodium acetate, pH 7.0, 60 μL of 50 mM $[Co(NH_3)_6]Cl_3$, and 660 μL of ethanol. The DNA was then precipitated, and the radioactive content of the supernatant was determined as previously described (Weiss et al., 1989). In one set of experiments, the reaction mixtures were directly applied to Dowex columns, and ion-exchange chromatography was performed to determine the nature of the enzyme-released photoproduct as previously described (Weiss et al., 1989).

Analysis of Released Bases. The UV photoproduct of thymine was separated from unmodified thymine by high-performance liquid chromatography (HPLC), following its release from UV-irradiated poly(dA-dT)·poly(dA-dT) by either endonuclease III or the human cell extract. The supernatant was lyophilized, dissolved in 200 μL of water, filtered, and injected into a 8 mm × 10 cm Waters C_{18} Radial-Pak cartridge in a Waters radial compression Z-module; 50 mM ammonium formate was used as an eluant at a rate of 1 mL/min. Unlabeled thymine was used as a marker. These fractions were collected at 0.5-min intervals, and their radioactivity was determined by liquid scintillation counting in 20% Biosolve (Beckman).

For analysis by thin-layer chromatography (TLC), the supernatants obtained after the enzymic reactions were dried, dissolved in methanol, and spotted on 20 × 20 cm aluminum-backed MN-cellulose plates (Merck) together with cold thymine. These were then developed in the lower layer of 2-propanol:H_2O (10:3) according to Cadet and Teoule (1971). The thymine spots were identified under a UV lamp; the lanes were then marked, cut into 1-cm strips, and counted in toluene–POPOP (Duker & Grant, 1980). Descending paper chromatographic analysis of released products was performed on Whatman 3MM paper in 1-butanol:formic acid:H_2O (10:2:15) (Breimer & Lindahl, 1985). The radioactivity was eluted from the paper and counted in 20% Biosolve (Beckman).

Chemical Synthesis, Purification, and Characterization of Damaged Thymine Moieties. 5-Bromo-6-hydroxy-5,6-dihydrothymine was synthesized according to Baudisch and Davidson (1925). Its identity was confirmed by mass spectroscopy. *cis*-Thymine hydrate was synthesized by reduction of 5-bromo-6-hydroxy-5,6-dihydrothymine with zinc in the presence of acetic acid (Cadet & Teoule, 1971). This compound was purified by repetitive HPLC on an 8 mm × 10 cm Waters C_{18} Radial-Pak cartridge in a Waters radial compression Z-module with a flow-rate gradient (0.8 mL/min to 2.8 mL/min in 20 min), 10 mM KH_2PO_4, pH 5.5, being used as eluant (Cadet et al., 1982). The *cis*-thymine hydrate was detected in the HPLC effluent by absorption of 214-nm light. The molecular weight of the compound was determined to be 144.2 by mass spectroscopy. *trans*-Thymine hydrate was synthesized by oxidation of *cis*-thymine hydrate with H_2O_2 to a mixture of *cis*- and *trans*-6-hydroperoxy-5,6-dihydrothymine, which were then reduced by KI to *cis*-thymine hydrate and *trans*-thymine hydrate (Cadet & Teoule, 1971). These were then purified with the HPLC system described above. Thymine glycol was synthesized by oxidation of thymine by $KMnO_4$ (Frenkel et al., 1981), followed by purification with HPLC using the flow-rate gradient system of Cadet et al. (1982). Its identity was confirmed by mass spectroscopy.

Photochemical Synthesis of Thymine Hydrates. Thymine hydrates were synthesized by irradiation of an aqueous 1 mM thymine solution under argon in a Hanovia photochemical reactor with a 450-W high-intensity Hg-vapor lamp and a borosilicate glass reaction vessel for 20 h. A total of 200 μL of [*methyl*-^{14}C]thymine (CEN, Saclay, France; 53.7 mCi/mmol, 108 μCi/mL) was added to the unlabeled thymine. Three photoproducts, two major and one minor, were separated by repetitive isocratic HPLC in 50 mM ammonium formate on an 8 mm × 10 cm Waters C_{18} Radial-Pak cartridge in a Waters radial compression Z-module. This yielded a single radioactive peak for each product.

The identification of these [^{14}C]thymine photoproducts was done by HPLC. The two major photoproducts obtained by irradiation of the thymine solution were cochromatographed with thymine, *cis*-thymine hydrate, *trans*-thymine hydrate, and thymine glycol on an 8 mm × 10 cm Waters C_{18} Radial-Pak cartridge in a Waters radial compression Z-module in 10 mM KH_2PO_4, pH 5.5, with the flow-rate gradient methodology of Cadet et al. (1982). The column was monitored with a 214-nm detector, and a fixed volume was collected in each fraction. Retention times were calculated according to Cadet et al. (1982).

Mass Spectroscopy Experiments. Positive fast atom bombardment (FAB) mass spectra (glycerol matrix) were obtained

Table I: Recovery of Thymine Photoproducts as Free Bases[a]

substrate	enzyme	% recovered radioactivity
unirradiated	endonuclease III	0.17
irradiated	endonuclease III	2.18
unirradiated	human enzyme	0.12
irradiated	human enzyme	0.41

[a] Radiolabeled poly(dA-dT)·poly(dA-dT) was irradiated as described for 1 h and incubated with 1.0 μg of *E. coli* endonuclease III for 30 min or with 30 μg of WI-38 cell extract for 1 h at 37 °C. The reaction mixtures were then applied to Dowex columns that were eluted with 1.35 mL of water. The radioactive content of the eluant was measured by liquid scintillation counting as described and the percentage of radioactivity from the columns calculated.

on a Finnegan Mat GC/EI-CI mass spectrometer (Model 4610B) equipped with an FAB attachment (Ion Tech, Ltd.). FAB-MS, m/z (relative intensity), positive mode: 237, $[M + \text{glycerol}]^+$; 145, $[M + H]^+$.

Results

The nature of the labeled material released from UV-irradiated poly(dA-dT)·poly(dA-dT) by the bacterial and human enzymes was investigated by ion-exchange chromatography. The results of a typical experiment are shown in Table I. Both endonuclease III and the human cell extract released radioactive material that did not bind to the Dowex resin. The net release by endonuclease III was 2.01% of the total UV-irradiated, radiolabeled material, and by the human enzyme it was 0.29%. Under these conditions, Dowex resin retains DNA and nucleotides, whereas free bases are eluted from the column. This demonstrates the released material to be free bases and that both the bacterial and the human activities acted as DNA glycosylases against the UV-irradiated substrate.

The material released from UV-irradiated poly(dA-dT)·poly(dA-dT) by *E. coli* endonuclease III was analyzed by HPLC. The freshly prepared substrate was irradiated for 60 min and incubated with 1.0 μg of endonuclease III for 30 min. Figure 1, an HPLC profile of the enzyme-released material, showed two distinct peaks of thymine photoproducts in addition to a minor peak identified as thymine. About 75% of the recovered radioactivity was present in these two major photoproduct peaks. The control supernatant from unirradiated DNA had but one minor peak at a position of one photoproduct, with no thymine release. This minor peak showed possible endogenous formation of a minor substrate moiety in the absence of irradiation. No significant release of radioactivity was observed in the absence of enzyme. Therefore, the enzyme-released photoproduct is separable from the unmodified thymine. A similar profile was obtained upon HPLC analysis of labeled material released by the human enzyme (data not shown). These results were confirmed by TLC, where the released photoproduct was also separated from thymine (data not shown). The photoproduct had an R_f of 0.71; the R_f of thymine was 0.81. Analysis by paper chromatography showed the enzyme-released photoproduct to be distinct from thymine (data not shown). In this system, the released photoproduct has an R_f of 0.65; that of thymine was 0.85.

To establish the identity of the enzyme-released material, the supernatant obtained after the incubation of UV-irradiated labeled polynucleotide with 2.0 μg of endonuclease III for 30 min was dried, dissolved in water, and cochromatographed with the two ^{14}C-labeled photochemically synthesized thymine photoproducts. The resulting HPLC profile is shown in Figure 2. It shows the thymine photoproducts released by endonuclease III eluted exactly at the same positions as the synthesized ^{14}C-labeled photoproducts. The exact same results were obtained upon chromatography of the photoproducts released by the human enzyme preparation (data not shown). Thus, the thymine photoproducts formed in UV-irradiated DNA were identical with those formed in thymine irradiated in aqueous solution. The [^{14}C]thymine photoproducts were then cochromatographed together with these marker compounds: *cis*-thymine hydrate, *trans*-thymine hydrate, thymine glycol (all chemically synthesized), and thymine. A flow-rate gradient was used with an eluent of 10 mM KH_2PO_4, pH 5.5. The retention time for each radioactive fraction (0.32 mL)

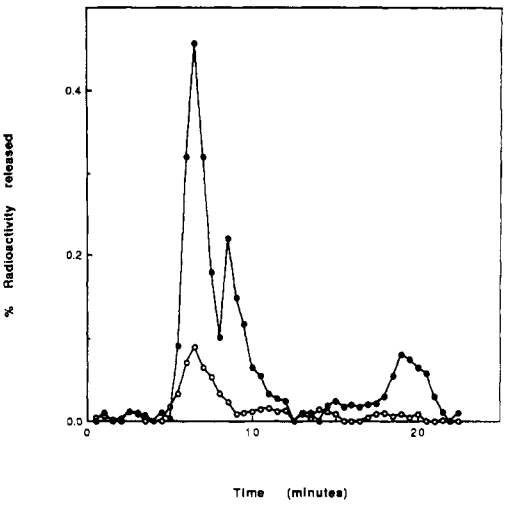

FIGURE 1: Separation of thymine photoproducts released from UV-irradiated poly(dA-dT)·poly(dA-dT) by endonuclease III from thymine. ^{3}H-Labeled poly(dA-dT)·poly(dA-dT) was irradiated with broad-spectrum UV light for 1 h and incubated with 1.0 μg of *E. coli* endonuclease III for 30 min at 37 °C. After the reaction, the ethanol-soluble fraction containing enzyme-released thymine photoproduct was dried under vacuum, dissolved in water, filtered, and analyzed by HPLC as described. The eluant was 50 mM ammonium formate (1 mL/min). The 0.5-mL fractions were counted in 20% Biosolve (Beckman). Enzyme-released ^{3}H-labeled material from irradiated (●) or unirradiated (○) substrates.

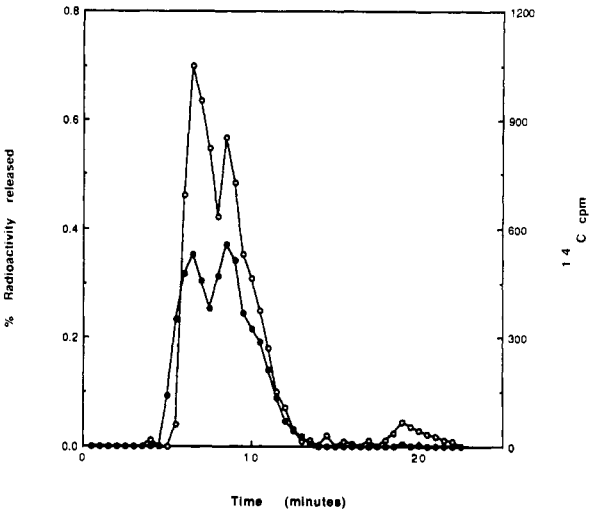

FIGURE 2: Cochromatography of ethanol-soluble ^{3}H-labeled photoproducts released from poly(dA-dT)·poly(dA-dT) by endonuclease III and the [^{14}C]thymine photoproducts. 2.0 μg of endonuclease III was incubated for 30 min with the substrate irradiated for 1 h. After the reaction, the ethanol-soluble fraction containing enzyme-released thymine photoproduct was dried under vacuum, dissolved in water, filtered, and analyzed by HPLC as described. The eluant was 50 mM ammonium formate (1 mL/min). Left ordinate: enzyme-released ^{3}H radioactivity (○). Right ordinate: ^{14}C-labeled photoproducts obtained by irradiation of thymine as described (●). The radioactivity of 0.5-mL fractions was determined by liquid scintillation counting.

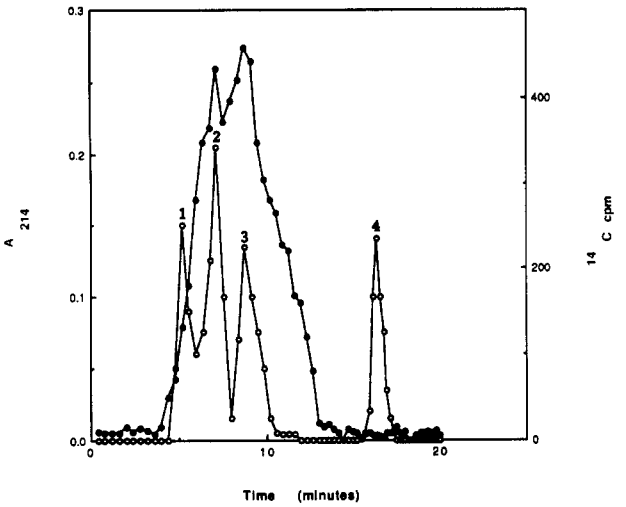

FIGURE 3: Identification of ^{14}C-labeled UV photoproducts of thymine by cochromatography with chemically synthesized marker compounds. The ^{14}C-labeled photoproducts of thymine, shown on the right ordinate (●), were injected with the following compounds (○): thymine glycol (peak 1), *trans*-thymine hydrate (peak 2), *cis*-thymine hydrate (peak 3), and thymine (peak 4). These were separated by HPLC using a flow-rate gradient (0.8–2.8 mL/min in 20 min). The eluant was 10 mM KH_2PO_4, pH 5.5. The column was monitored at 214 nm (plotted in arbitrary units), and 0.32-mL fractions were collected and counted in 20% Biosolve. The retention time for each fraction was then calculated.

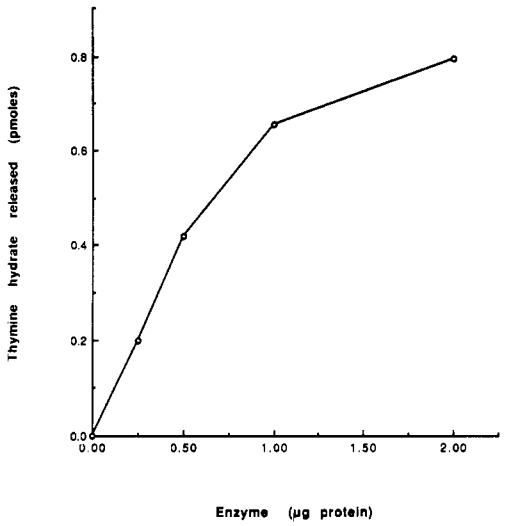

FIGURE 4: v vs $[E]_t$ plot of release of thymine hydrates from irradiated poly(dA-dT)·poly(dA-dT) by *E. coli* endonuclease III. ^3H-Labeled poly(dA-dT)·poly(dA-dT), irradiated for 1 h, was incubated for 30 min at 37 °C with increasing amounts of enzyme. The reaction mixture was precipitated as described, and the ethanol-soluble radioactivity was determined by liquid scintillation counting. Each point represents the average of three independent determinations.

was determined. The results are shown in Figure 3. Comparisons of the HPLC profiles of the thymine photoproducts and of the chemically synthesized reference compounds show the [^{14}C]thymine photoproducts to be *cis*-thymine hydrate and *trans*-thymine hydrate. No thymine glycol was released from the irradiated substrate. These results demonstrate enzymic release of both thymine hydrates from UV-irradiated poly(dA-dT)·poly(dA-dT) by endonuclease III and the human cell extract.

The kinetics of release of thymine hydrates by endonuclease III were examined. Increasing amounts of endonuclease III were incubated for 30 min at 37 °C with the UV-irradiated substrate. The corresponding v vs $[E]_t$ plot is shown in Figure 4. Enzymic excision of thymine hydrates increased in a linear

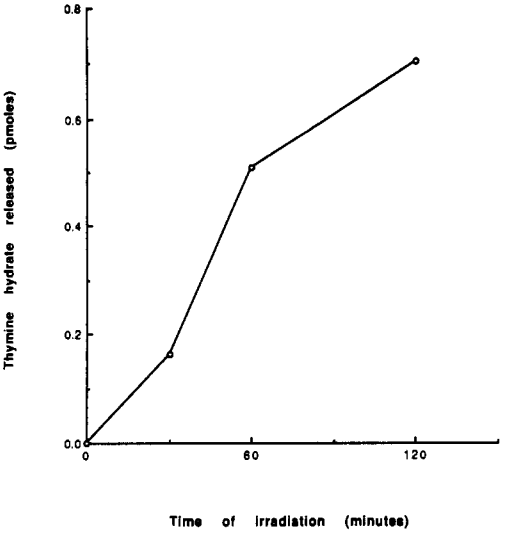

FIGURE 5: v vs [S] plot of release of thymine hydrates from irradiated poly(dA-dT)·poly(dA-dT) by endonuclease III. The radiolabeled substrate was irradiated as described for the indicated times and incubated for 30 min at 37 °C with 1.0 μg of the enzyme. The reaction mixture was precipitated as described, and the ethanol-soluble radioactivity was determined. Each point represents the average of three independent determinations.

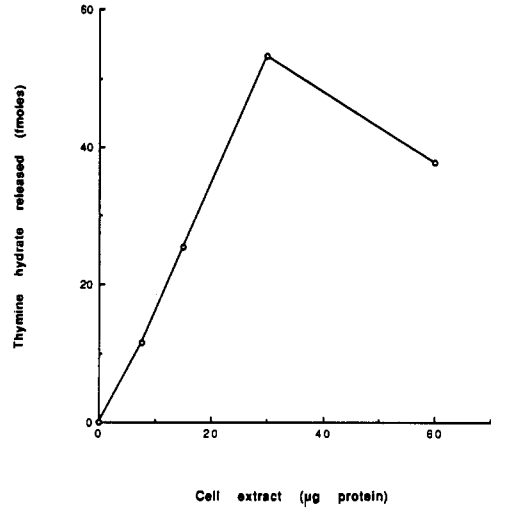

FIGURE 6: v vs $[E]_t$ plot of release of thymine hydrates from irradiated poly(dA-dT)·poly(dA-dT) by WI-38 cell extracts. ^3H-Labeled poly(dA-dT)·poly(dA-dT) was irradiated for 1 h as described and incubated for 1 h at 37 °C with increasing amounts of cell protein. The release of thymine hydrates into the ethanol-soluble fraction was measured by HPLC in 50 mM ammonium formate (1 mL/min) as described. The fractions (0.5 mL) were counted in 20% Biosolve. Each point represents the average of two independent determinations.

manner with quantity of enzyme up to 1.0 μg of endonuclease III per reaction mixture. No release was obtained in the absence of enzyme. The specific activity, as determined from the initial portion of the curve, was 0.028 pmol of thymine hydrates released per microgram of protein per minute. In the experiment shown in Figure 5, 1.0 μg of endonuclease III was incubated with substrate that had been irradiated with increasing doses of UV light. Photoproduct release increased almost linearly over the range of irradiation used. Thus, the yield of thymine hydrates in DNA increased with dose. At the maximum dose, about 1.5% of substrate radioactivity was released into the ethanol-soluble fraction under conditions of enzyme excess.

The human activity against DNA thymine hydrates was then characterized in a similar manner. Increasing concentrations of WI-38 cell extract were incubated for 1 h with the

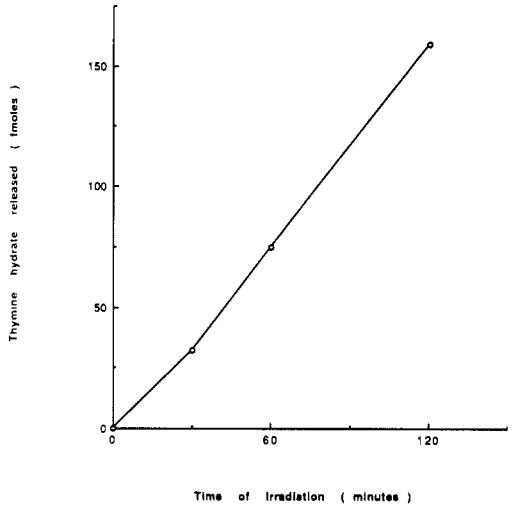

FIGURE 7: v vs [S] plot of release of thymine hydrates from irradiated poly(dA-dT)·poly(dA-dT) by WI-38 cell extracts. The radiolabeled substrate was irradiated for the indicated times and incubated for 1 h at 37 °C with 30 μg of cell protein. The release of thymine hydrates into the ethanol-soluble fraction was measured by HPLC. Each point represents the average of two independent determinations.

UV-irradiated substrate. The ethanol-soluble released material was analyzed by HPLC. The corresponding v vs $[E]_t$ plot is shown in Figure 6. Enzymic excision of thymine hydrates increased with quantity of extract up to 30 μg of cell protein per reaction mixture. The specific activity, as determined from the initial portion of the curve, was 0.06 fmol of thymine hydrates released per microgram of protein per minute. When the cell extract was incubated for 1 h with substrate polynucleotide irradiated with increasing doses of UV light, there was a concomitant increase in enzymic release of ^{3}H-labeled material, shown in Figure 7. After 1 h of substrate irradiation, 0.17% of substrate radioactivity was recovered as thymine hydrate. There was no saturation of release of the DNA photohydrates, even following 2 h of substrate irradiation.

Discussion

The effects of UV irradiation on living cells include cell death, mutation, and neoplastic transformation (Harm, 1980). Such irradiation results in a variety of photoproducts in cellular DNA. These include pyrimidine dimers and 6–4 photoproducts (Patrick & Rahn, 1976). Pyrimidine photohydrates are formed in lesser yields (Fisher & Johns, 1976; Patrick & Rahn, 1976). It has been recently demonstrated that both cytosine and uracil photohydrates are formed in UV-irradiated DNA and that these are excised by bacterial and mammalian redoxyendonucleases (Boorstein et al., 1989). The present experiments show the formation, in UV-irradiated poly(dA-dT)·poly(dA-dT), of yet another type of pyrimidine photoproduct that is excised by such enzymes. Since this substrate has an alternating purine–pyrimidine sequence, formation of both pyrimidine dimers and 6–4 photoproducts is unlikely; this was confirmed by appropriate controls. A photoproduct formed by covalent linkage of adenines to adjacent thymines has been isolated from UV-irradiated oligodeoxyribonucleotides and DNA (Bose et al., 1983; Bose & Davies, 1984). Activities that incise DNA at purine photoproduct sites have been detected (Duker & Gallagher, 1986; Gallagher & Duker, 1986, 1989). Ion-exchange Dowex chromatography was therefore initially used to establish the nature of the enzyme-released material. Since all material other than free bases is retained by the Dowex resin, the results, shown in Table I, demonstrate that the labeled materials released from DNA by both the bacterial and human enzymes are free bases

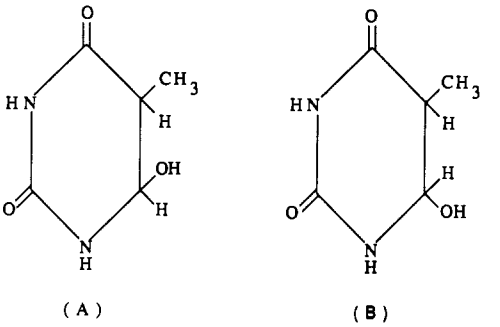

FIGURE 8: Structures of (A) *cis*-thymine hydrate and (B) *trans*-thymine hydrate.

(Duncan et al., 1978). No material released as nucleotide was detected in any of the assay systems utilized here. Therefore, the labeled base photoproducts excised from UV-irradiated poly(dA-dT)·poly(dA-dT) by *E. coli* endonuclease III and extracts of WI-38 cells consist only of monomeric thymine base derivatives.

A base release assay was done to characterize *E. coli* endonuclease III and the human activity against the radiolabeled, UV-irradiated poly(dA-dT)·poly(dA-dT) and to examine their mechanism of action. WI-38 cells were chosen as the human enzyme source because of their high levels of endonuclease activity against non-pyrimidine dimer products of UV-irradiated DNA (Duker & Teebor, 1975). While more than one type of redoxyendonuclease has been described in mammalian cells, only one such enzyme is active against a linear DNA substrate (Kim & Linn, 1989). Therefore, this type of activity is the one measured in the WI-38 extract. This assay involved use of $[Co(NH_3)_6]Cl_3$ as a DNA precipitating agent. Without its inclusion, precipitation of poly(dA-dT)·poly(dA-dT) was incomplete, and the base release assay could not be performed. The thymine photoproducts released from DNA were compared with characterized markers, synthesized by both chemical and photochemical means. Three chromatographic systems were used: TLC, paper chromatography, and HPLC.

Analysis of the released photoproducts by TLC showed them to be separable from thymine. The photoproduct had an R_f of 0.71, while the R_f of thymine was 0.81. These separations were comparable to those obtained by Cadet and Teoule (1971). Analysis by paper chromatography yielded a similar separation. In this system, the excised photoproduct has an R_f of 0.65, while that of thymine was 0.85. These separations were comparable to those obtained by Breimer and Lindahl (1985). Therefore, the released photoproducts had chromatographic properties reported by other investigators for the thymine hydrates in these systems.

HPLC analysis of released photoproducts also showed them to be distinct from free thymine, although some free thymine was recovered as well in these chromatograms (Figure 1). The substrate photoproducts were then identified by their cochromatography with photochemically synthesized [^{14}C]thymine photoproducts (Figure 2). The [^{14}C]thymine photoproducts were identical with the enzyme-released DNA ^{3}H-labeled photoproducts. This was true of ^{3}H-labeled materials excised from substrate DNAs both by endonuclease III and by the human activity. Their identity was then confirmed by their cochromatography with chemically synthesized *cis*-thymine hydrate and *trans*-thymine hydrate (Figure 3). Their structures are shown in Figure 8.

Previous studies of pyrimidine photohydrates have shown them to have differing stabilities. Uracil hydrate is stable both as a free base in solution and in DNA; cytosine hydrate, although stable while in DNA, rapidly reverts to cytosine in

neutral aqueous solution (Boorstein et al., 1989). The recovery of both thymine hydrates in these assays indicates them to be essentially stable in solution. This stability of thymine hydrates, with each type having a half-life of 24 h in neutral solution, resembles that of uracil hydrate rather than that of cytosine hydrate (Fisher & Johns, 1973). While some free thymine was occasionally recovered from the reaction mixtures, indicating some reversion, the general stability of thymine hydrates in solutions should facilitate further studies of their excision–repair from DNA.

It has been demonstrated that 280 nm is the optimal wavelength for formation of DNA cytosine hydrate, the major substrate for *E. coli* endonuclease III and the human activity (Weiss & Duker, 1987; Gallagher et al., 1989a). Because of the low yield of thymine hydrates in irradiated DNA, high doses of broad-spectrum UV light were required for these experiments. This is in contrast with the relatively abundant formation of cytosine hydrates in poly(dG)-dC)·poly(dG-dC) and uracil hydrates in poly(dA-dU)·poly(dA-dU) by monochromatic 280-nm light (Weiss et al., 1989; Ganguly & Duker, 1990). The quantum yield of thymine photohydration in aqueous solution is 3 orders of magnitude lower than that of uracil photohydration (Fisher & Johns, 1973). Uracil, in turn, is an order of magnitude more susceptible to destruction by UV radiation than cytosine (Kochetkov & Budovskii, 1972; Fisher & Johns, 1976). This is paralleled by the photoreactivity of these bases in DNA; probes of synthetic polydeoxyribonucleotides with endonuclease III indicate photohydration of dU to be greater than that of dC, which in turn far exceeds that of dT (Boorstein et al., 1989; Weiss et al., 1989; Ganguly & Duker, 1990). The photohydration of 5-methylcytosine in DNA has not been examined.

While the reduced rates of enzymic excision of thymine hydrates from poly(dA-dT)·poly(dA-dT) reflect the documented lower photochemical yield, the possibility that greater quantities of such photohydrates are produced and removed with lesser efficiency cannot be totally excluded. While the poly(dA-dT)·poly(dA-dT) is initially double stranded, the high fluences of irradiation could alter the substrate conformation, resulting in reduced enzyme binding. Endonuclease III requires native DNA as substrate (Breimer, 1983; Breimer & Lindahl, 1984; Higgins et al., 1987). Although DNA thymine glycol is a substrate for endonuclease III, there was no excision of that base from extensively oxidized and denatured poly(dA-dT)·poly(dA-dT) (Higgins et al., 1987). This might be reflected in the low turnover numbers of both endonuclease III and the human WI-38 activity for DNA thymine hydrates. Purified endonuclease III released far more thymine hydrates than the WI-38 cell preparation; the same result was obtained when releases of cytosine hydrates were compared between the pure bacterial enzyme and a sonicate of human HeLa cells (Boorstein et al., 1989). These differences were not present when the purified human and bacterial enzymes were compared (Weiss et al., 1989). However, turnover numbers of endonuclease III and HeLa cell sonicate are considerably higher for excision of both cytosine and uracil hydrates from synthetic polydeoxyribonucleotides than those determined here for thymine hydrates (Boorstein et al., 1989). This may be due to substrates that are less denatured than the one we studied, resulting from lower fluences of UV irradiation.

The apparent low yields of thymine hydrates in irradiated DNA indicate them to be minor chemical products as compared with cytosine hydrates, and certainly when compared with pyrimidine dimers or 6–4 photoproducts. This is consistent with the finding of cytosines as the major or exclusive sites of incision of UV-irradiated DNAs by endonuclease III or the human activity with sequencing techniques (Doetsch et al., 1986, 1987; Weiss & Duker, 1986, 1987; Gallagher et al., 1989a,b; Weiss et al., 1989). If pyrimidine photohydration in synthetic polydeoxyribonucleotides reflects relative yields of formation in cellular DNA, the fluences required to induce thymine hydrates in any significant yields would be far in excess of the range of cell survival, and these products would be unlikely to be of biological consequence. However, this may not be the case. Stretches of poly(dG-dT)·poly(dA-dC) are present in actively transcribing regions of eukaryotic cellular DNAs (Kim et al., 1981; Hamada & Kakunaga, 1982; Hamada et al., 1982). These could be preferential sites of pyrimidine photohydration. One study using an extract from human HeLa cells showed nicking of a UV-irradiated fragment from the pUC18 plasmid at many thymine sites (Doetsch et al., 1988). This could indicate differing susceptibilities of specific sequences to different photochemical modifications. Sensitizing or other factors within the cell may lead to a different distribution of pyrimidine photoproducts upon UV irradiation. With a chemical degradation assay, thymine glycol was shown to be formed in DNA following irradiation of HeLa cells by various monochromatic *uvB* and *uvC* fluences (Hariharan & Cerutti, 1977). We found no thymine glycol to be excised from UV-irradiated poly(dA-dT)·poly(dA-dT), even at the high mixed-wavelength fluences in these studies. Resolution of these questions requires comparisons of yields of the different altered pyrimidine moieties between different sequences of irradiated purified DNAs and those of cultured cells.

These results that thymine hydrates are formed in UV-irradiated DNA and recognized by endonuclease III and the human activity are consistent with the known mechanisms of action of these enzymes. Thymine lesions recognized by these activities have, in common, a loss of the 5,6-double bond, resulting in nonplanar structures (Weiss & Grossman, 1987; Sancar & Sancar, 1988; Wallace, 1988). A modified thymine derivative was released by endonuclease III from DNA that had been γ-irradiated under hypoxic conditions; this product was definitively identified, although thymine hydrate was proposed as a possibility (Breimer & Lindahl, 1985). Our results indicate that *cis*-thymine hydrate and *trans*-thymine hydrate are both produced in UV-irradiated DNA. This highly irradiated DNA substrate is sufficiently stable, so these photoproducts are recognized and excised by both endonuclease III and the human activity. It appears that such thymine hydrates are sufficiently stable in DNA to cause biological consequences if left unrepaired. These could include interference with transcription, inhibition of replication, or mutagenesis. Such deleterious effects would necessitate the repair of these altered DNA thymine moieties.

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