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Formation and Stability of Repairable Pyrimidine Photohydrates in DNA†

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ABSTRACT: Ultraviolet irradiation of poly(dG-dC) and poly(dA-dU) in solution produces pyrimidine hydrates that are repaired by bacterial and mammalian DNA glycosylases [Boorstein et al. (1989) *Biochemistry 28*, 6164–6170]. *Escherichia coli* endonuclease III was used to quantitate the formation and stability of these hydrates in the double-stranded alternating copolymers poly(dG-dC) and poly(dA-dU). When poly(dG-dC) was irradiated with 100 kJ/m^2 of 254-nm light at pH 8.0, 2.2% of the cytosine residues were converted to cytosine hydrate (6-hydroxy-5,6-dihydrocytosine) while 0.09% were converted to uracil hydrate (6-hydroxy-5,6-dihydrouracil). To measure the stability of these products, poly(dG-dC) was incubated in solution for up to 24 h after UV irradiation. Cytosine hydrate was stable at 4 °C and decayed at 25, 37, and 55 °C with half-lives of 75, 25, and 6 h. Uracil hydrate produced in irradiated poly(dA-dU) was stable at 4 °C and at 25 °C and decayed with a half-life of 6 h at 37 °C and less than 0.5 h at 55 °C. Uracil hydrate and uracil were also formed in irradiated poly(dG-dC). These experiments demonstrate that UV-induced cytosine hydrate may persist in DNA for prolonged time periods and also undergo deamination to uracil hydrate, which in turn undergoes dehydration to yield uracil. The formation and stability of these photoproducts in DNA may have promoted the evolutionary development of the repair enzyme endonuclease III and analogous DNA glycosylase/endonuclease activities of higher organisms, as well as the development of uracil–DNA glycosylase.

The mutagenicity and carcinogenicity of ultraviolet (UV) radiation have been attributed to the effects of base modifications formed in DNA as a consequence of such radiation (Witkin, 1976; Harm, 1980; Hall & Mount, 1981; Hutchinson, 1987). The most readily formed UV-induced base modifications are the cyclobutane pyrimidine dimers and the pyrimidine 6–4 photoproducts (Setlow & Carrier, 1963; Fisher & Johns, 1976; Patrick & Rahn, 1976; Franklin & Haseltine, 1986; Mitchell & Mairn, 1989). However, all the biologic consequences of UV irradiation cannot result from the effects of these lesions. In particular, mutations formed at cytosine residues (Brash & Haseltine, 1982; Lebkowski et al., 1985; Glazer et al., 1986; Schaaper et al., 1987; Brash et al., 1987) that are not adjacent to other pyrimidines cannot be ascribed to either of the dimeric photoproducts. Instead, these mutations might result from the UV-induced modification of single cytosine residues.

The most readily formed UV-induced modification of single cytosine residues is the photohydrate resulting from addition of water to the 5,6 double bond (Kochetkov & Budovskii, 1972; Fisher & Johns, 1976). UV-induced formation of this mod-

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ification has been definitively demonstrated in cytosine, deoxycytidine, and deoxycytidine monophosphates (Kochetkov & Budovskii, 1972; Fisher & Johns, 1976). Hydrates of these compounds are unstable and rapidly revert by eliminating a molecule of water (Kochetkov & Budovskii, 1972; Fisher & Johns, 1976). The formation of cytosine hydrate in DNA has been inferred from a variety of measurements including a decrease in the UV absorbance of DNA following irradiation (Setlow & Carrier, 1963), the formation of tritiated water after irradiation of DNA containing tritiated cytosine (Grossman & Rogers, 1968), and a change in the coding properties of poly(dC) as a template for in vitro DNA polymerase reactions after UV irradiation (Lecomte et al., 1981).

Experiments using the *Escherichia coli* repair enzyme endonuclease III also indicated that UV irradiation yielded modification of single cytosine residues. The enzyme contains two activities that act sequentially: a DNA glycosylase that catalyzes hydrolysis of the *N*-glycosyl bond releasing the modified pyrimidine base and an AP (apurinic/apyrimidinic) endonuclease that catalyzes cleavage of the phosphodiester bond of the resulting abasic (AP) site by a β-elimination mechanism (Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984; Bailly & Verly, 1987). Endonuclease III incises chemically oxidized or γ-irradiated DNA at thymine residues, releasing thymine glycol and other oxidized thymine derivatives (Breimer, 1983; Doetsch et al., 1986; Higgins et al., 1987; Kow & Wallace, 1987; Wallace, 1988). When UV-irradiated DNA was exposed to endonuclease III, incision occurred at cytosine residues, and cytosine-derived material was released from the UV-irradiated double-stranded alternating copolymer poly(dG-[^{3}H]dC) (Doetsch et al., 1986; Weiss & Duker, 1986, 1987).

We demonstrated that cytosine hydrate was formed in DNA (Figure 4, compound II) by identifying the material released by endonuclease III from UV-irradiated poly(dG-[^{3}H]dC) by HPLC analysis (Boorstein et al., 1989). The compound proved to be cytosine. We explained this finding by suggesting that the enzyme recognized and released cytosine hydrate from UV-irradiated DNA. Once released from the backbone, the photohydrate rapidly eliminated water and reverted to cytosine. To prove that the enzyme recognized pyrimidine photohydrates in DNA, we incubated the UV-irradiated double-stranded alternating copolymer poly(dA-[^{3}H]dU) with endonuclease III and demonstrated the release of intact uracil hydrate from the copolymer backbone. Uracil hydrate is known to eliminate water much less readily than cytosine hydrate. Similar results were obtained when the DNA was incubated with extracts of HeLa cells. Thus, we concluded that cytosine hydrate was formed in DNA by UV irradiation and that it was a substrate for both bacterial and mammalian DNA glycosylase activities.

These results, together with those of earlier experiments, suggested that hydrates of cytosine in DNA were much more stable than hydrates of free cytosine, deoxycytidine, and deoxycytidine monophosphate. To prove this hypothesis, we undertook measurements of the formation and stability of cytosine hydrate in UV-irradiated poly(dG-dC). The availability of *E. coli* endonuclease III (Asahara et al., 1989) purified from an efficient expression system has made it feasible to use the enzyme as a reagent with which to measure the formation of pyrimidine photohydrates in UV-irradiated DNA. The double-stranded alternating copolymer was used in these studies because it assumes the B-DNA conformation in salt solutions (Patel et al., 1979; Pohl & Jovin, 1972) and is therefore a good model for studying the properties of these photohydrates in B-DNA. It is particularly suited for this study because the alternating purine–pyrimidine sequence precludes the formation of dimeric pyrimidine photoproducts. Such photoproducts interfere with the action of DNA glycosylases (Duker et al., 1981).

Experimental Procedures

Materials

Enzyme. *E. coli* endonuclease III purified from the cloned *nth* gene (Asahara et al., 1989) was stored in a 5 mg/mL solution (100 mM potassium phosphate, pH 6.6, 50% glycerol). The 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 biology grade BSA (BRL), and 10% glycerol.

Radionucleotides. [5-^{3}H]-2′-Deoxycytidine 5′-triphosphate (dCTP) (28.2 Ci/mmol) was purchased from New England Nuclear. [5-^{3}H]-2′-Deoxyuridine 5′-triphosphate (dUTP) (28.2 Ci/mmol) was purchased from Amersham.

Uracil Hydrate. 6-Hydroxy-5,6-dihydrouracil (uracil hydrate) was isolated from a UV-irradiated solution of a 1 mM aqueous solution of uracil as previously described (Boorstein et al., 1989).

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 with a nick-translation kit (Boorstein et al., 1989) (NEN). The nick-translated copolymer was purified on a Sephadex G-50 minispin column (Worthington).

UV Irradiation. Nick-translated poly(dG-[^{3}H]dC) or poly(dA-[^{3}H]dU) in TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0) was irradiated by use of an unfiltered double fixture containing two General Electric 15-W germicidal bulbs (G15TA) with a primary output of 254 nm (Carrier & Setlow, 1971; Duker & Teebor, 1975). All samples were irradiated in weighing boats on ice.

Enzymatic Assay of ^{3}H-Containing Photoproducts in Irradiated ^{3}H-Containing Copolymer. To determine the pyrimidine photohydrate content of irradiated copolymers, 0.1 μg of copolymer was incubated with 1 μg of endonuclease III in a final volume of 12 μL for 30 min at 37 °C. This methodology was used by Breimer and Lindahl (1985) to quantify the formation of radiation-induced pyrimidine derivatives. The final reaction mixture contained 50 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, and 0.1 mg/mL BSA. The reaction was stopped by the addition of 12 μL of BSA and 50 μL of cold acetone to the mixture. Acetone-insoluble material was separated by centrifugation (Boorstein et al., 1989), and the acetone-soluble material was dried under vacuum, resuspended in 200 μL of water, and analyzed for the presence of [^{3}H]cytosine and [^{3}H]uracil hydrate by HPLC.

These assay conditions had been determined by incubating 0.1 μg of irradiated poly(dG-[^{3}H]dC) (100 kJ/m^2) with 0.001–4 μg of endonuclease III and measuring the release of [^{3}H]cytosine from the UV-irradiated copolymer. Release was half-maximal at enzyme concentrations of 0.1 μg per reaction and plateaued above 0.4 μg per reaction (Figure 1). Therefore, in subsequent assays determining the amount of photohydrates in the copolymers, an amount of enzyme ten times that which gave half-maximal release was placed in the reaction mixture. This large excess of enzyme ensured that the amount of radioactive material released equaled the actual amount of photohydrate formed in the UV-irradiated copolymer.

HPLC Analysis of Enzymatically Released ^{3}H-Containing Material from Poly(dG-[^{3}H]dC) and Poly(dA-[^{3}H]dU).

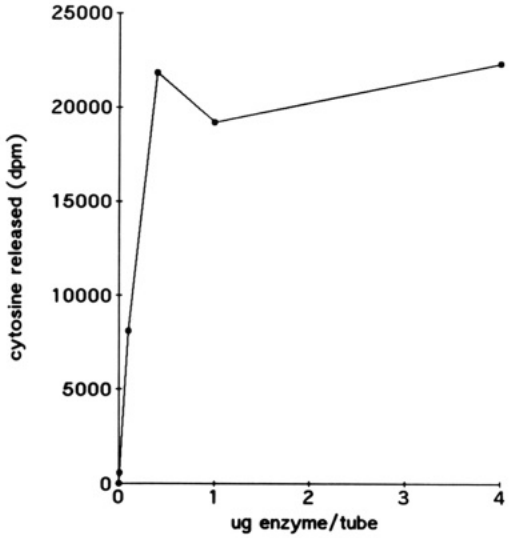

FIGURE 1: v vs $[E]_t$ plot of cytosine release from poly(dG-[^{3}H]dC). Increasing amounts of endonuclease III were incubated with 0.1 μg of irradiated poly(dG-[^{3}H]dC), and cytosine release in 30 min was measured by HPLC.

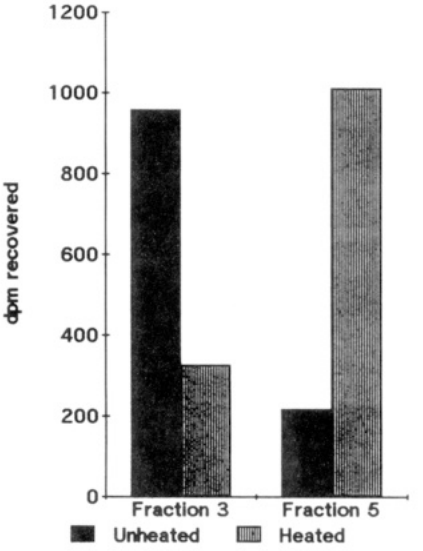

FIGURE 2: TLC analysis of ^{3}H-containing acetone-soluble products released from poly(dG-[^{3}H]dC) by endonuclease III. Half the sample was heated prior to analysis. Radioactivity coeluting with uracil hydrate (fraction 3, R_f = 0.23–0.4) or uracil (fraction 5, R_f = 0.45–0.52) was determined.

Samples were analyzed on a 5-μm ultrasphere ODS column using 50 mM ammonium formate as the eluant at a rate of 1 mL/min. Between runs, the column was washed for 15 min with methanol and equilibrated with ammonium formate for 20 min. The elution of material containing radioactivity was monitored through the use of a Radiomatic Flo-One in-line radioactivity detector. Uracil and cytosine were added to the samples, and the elution of these internal standards was monitored by UV absorbance. The retention time of added nonradioactive marker uracil hydrate was determined by collecting HPLC fractions and analyzing them by TLC.

Thin-Layer Chromatography (TLC). Samples of enzymatically released material were initially separated by HPLC and then analyzed by TLC (Teoule et al., 1974; Cadet et al., 1976; Boorstein et al., 1989). The samples were spotted onto silica gel plates, and the chromatographs were developed with the lower phase of chloroform/methanol/water (4:2:1) to which 5 mL of methanol had been added for each 100 mL of organic phase until the solvent front reached 1 cm from the top of the plate. The plates were air-dried and then heated. Heating converts uracil hydrate, which does not absorb UV light, to uracil, which does. Following chromatography, regions coeluting with the UV fluorescent markers were outlined, scraped into scintillation vials, and analyzed by liquid scintillation counting.

Enzymatic Assay of [^{3}H]Uracil in Irradiated Poly(dG-[^{3}H]dC). To determine the uracil content of irradiated poly(dG-dC), 0.1 μg of copolymer was incubated with 60 units of calf thymus uracil–DNA glycosylase (1 unit of enzyme releases 1 pmol of uracil per minute from PBS DNA) in a final volume of 200 μL for 90 min at 37 °C. The final reaction mixture contained 50 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, and 0.1 mg/mL BSA. The reaction was stopped by the addition of BSA and cold acetone. Acetone-insoluble material was separated by centrifugation, and the acetone-soluble material was dried under vacuum, resuspended in 200 μL of water, and analyzed for the enzymatic release of [^{3}H]uracil by HPLC.

Results

Demonstration of Formation of Cytosine Hydrate and Uracil Hydrate in Poly(dG-dC). We have previously demonstrated that the major cytosine photoproduct formed in poly(dG-dC) and recognized by endonuclease III is cytosine hydrate (Boorstein et al., 1989). Once the cytosine hydrate is released from the copolymer, it nonenzymatically loses water and reverts to cytosine.

To determine whether uracil hydrate residues are also formed following the irradiation of poly(dG-dC), 0.1 μg of poly(dG-[^{3}H]dC) (9–13 × 10^6 dpm/μg) was irradiated (100 kJ/m^2) and incubated with endonuclease III. The acetone-soluble products of this reaction were analyzed by HPLC. In three experiments, 0.09% ± 0.03% of the initial radioactivity was recovered as uracil hydrate, while 2.2% ± 0.6% was recovered as cytosine.

This calculation of the percentage of initial radioactivity recovered as cytosine includes a correction factor to account for the loss of ^{3}H during the formation and dehydration of cytosine hydrate. DeBoer and Johns (1970) demonstrated a loss of 50% of the tritium from UV-irradiated [^{3}H]Cyd and [^{3}H]CMP post UV irradiation. They attributed this loss primarily to tritium exchange with water resulting from labilization of the carbon–tritium bond of the photohydrate and, to a lesser extent, from the elimination of [^{3}H]H_2O during dehydration of the photohydrate. We therefore assume that the specific activity of the enzymatically released cytosine is half the specific activity of the cytosine hydrate within the copolymer.

To confirm that the ^{3}H-containing material coeluting with uracil hydrate was authentic uracil hydrate, the acetone-soluble products of the reaction of 0.2 μg of poly(dG-dC) were separated by HPLC using ammonium formate as an eluant (1 mL/min). Fractions were collected at 0.5-min intervals, and the fraction eluting with the retention time of uracil hydrate and the two adjacent fractions were pooled. Half the pooled sample was heated under conditions in which uracil hydrate is converted to uracil. The other half was reserved at 4 °C. When the samples were analyzed by TLC, the unheated material coeluted with uracil hydrate. The heated material coeluted with uracil (Figure 2). These experiments suggest that uracil hydrate can be formed from cytosine in poly(dG-dC) following UV irradiation.

An estimate of the yield of the UV-induced formation of pyrimidine hydrates in the copolymer was determined by calculating the reaction cross section from the concentration

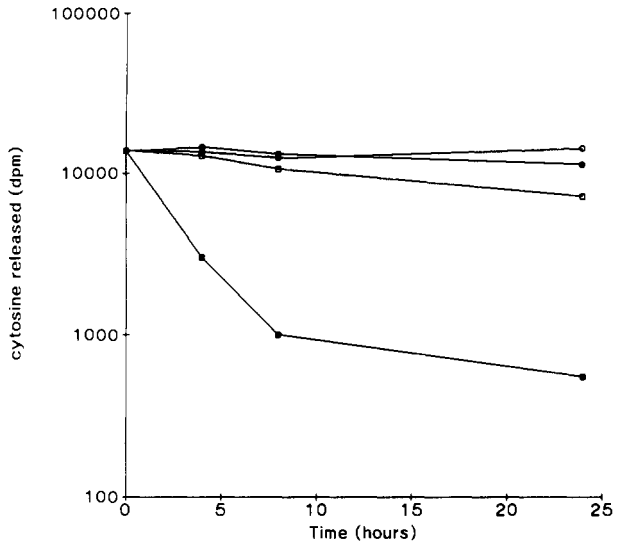

FIGURE 3: Stability of cytosine hydrate in poly(dG-[^{3}H]dC). Poly(dG-[^{3}H]dC) was irradiated and then incubated at 4 °C (open circles), 25 °C (closed circles), 37 °C (open squares), and 55 °C (closed squares). After 0, 4, 8, or 24 h, the DNA was incubated with endonuclease III and cytosine release was determined.

of starting material, the concentration of product, and the average exposure to UV light according to the method described by Jericevic et al. (1982). The reaction cross section was found to be 0.001 $cm^2/\mu E$.

Stability of Cytosine Hydrate in Poly(dG-dC). Poly(dG-dC) in solution was irradiated and then incubated at 4, 25, 37, or 55 °C. After 0, 4, 8, or 24 h, the polymer was incubated with endonuclease III and the amount of cytosine released was determined. The amount of cytosine released decreased with time and increasing temperature (Figure 3) with rate constants of 0.009 ± 0.003 h^{-1} at 25 °C, of 0.02 ± 0.002 h^{-1} at 37 °C, and of 0.12 ± 0.05 h^{-1} at 55 °C. Incubation of unirradiated poly(dG-dC) at these temperatures did not by itself produce cytosine hydrate in DNA. To demonstrate that incubation of the copolymer did not affect its ability to serve as an enzyme substrate, the copolymer was first incubated for up to 24 h at temperatures from 4 to 55 °C, UV irradiated, and then mixed with enzyme. The same amount of cytosine was enzymatically released from the copolymer incubated at these temperatures prior to irradiation as from the unincubated copolymer.

Representative half-life values for the decay of cytosine hydrate were determined from the data shown. The cytosine hydrate was stable at 4 °C and decayed with half-lives of 75, 25, and 6 h at 25, 37, and 55 °C.

The temperature dependence of this reaction was further expressed as a function of the log of the reaction rate (log k) versus the reciprocal of the temperature following the Arrhenius equation. The activation energy of the reaction was determined to be 16 kcal/mol from the slope ($n = 3$, $r^2 = 0.99$) of this line.

Stability of Uracil Hydrate in Poly(dA-dU) and Poly(dG-dC). The stability of uracil hydrate in UV-irradiated poly(dA-dU) was determined in a manner similar to the determination of cytosine hydrate stability in poly(dG-dC). Uracil hydrate formed in poly(dA-dU) by 100 kJ/m^2 of UV light was stable at 4 and 25 °C but decayed with a half-life of 6 h at 37 °C and less than 0.5 h at 55 °C. The activation energy for this decay was estimated to be about 30 kcal/mol. However, uracil hydrate formed in irradiated poly(dA-dU) may inadequately model uracil hydrate formed in DNA, since the uracil hydrate is formed opposite adenine, not guanine, and since normal DNA does not contain uracil.

To estimate the stability of uracil hydrate formed from cytosine in poly(dG-dC), a more relevant model, two additional experiments were performed. First, irradiated poly(dG-dC) was incubated with a large excess of uracil-DNA glycosylase and the amount of uracil release was measured. Immediately after irradiation, uracil could not be detected. (The detection limit was 0.01% of the initial radioactivity of the copolymer.) After a 24-h incubation at 37 °C, 0.03–0.37% of the initial radioactivity was recovered as uracil. This accounts for one-third to one-half of the uracil hydrate initially formed in the copolymer.

Second, irradiation poly(dG-dC) was incubated for 24 h at 37 °C and then incubated with endonuclease III. The amounts of radioactivity recoverable as cytosine and uracil hydrate were determined. After this 24-h incubation, the ratio of cytosine hydrate to uracil hydrate in three determinations averaged 22.9 ± 13.8. This ratio was essentially unchanged from the ratio at 0 h of 25.1 ± 5.5. While the standard deviations in this experiment are large and widened with time due to the large background of cytosine, the lack of change in the cytosine/uracil hydrate ratio indicates that uracil hydrate in poly(dG-dC) is neither markedly more nor markedly less stable than cytosine hydrate.

Discussion

Cytosine hydrate is the principal repairable nondimeric photoproduct of cytosine produced by UV irradiation of DNA. At physiologic temperature (37 °C) and pH (7.6), cytosine hydrate in poly(dG-dC) decayed with a half-life of 18 h. In contrast, the half-lives of the photohydrates of cytosine, deoxycytidine, CMP, and dCMP (Fisher & Johns, 1976) at 20–30 °C are about 1% as long.

Empirically, the rate of this type of reaction (Weston & Schwarz, 1972) can be expressed by the Arrhenius equation as a function of a logarithmic decay term dependent on the activation energy for the reaction and inversely dependent on temperture and an independent "A factor" or "frequency factor". The frequency factor has been mathematically associated with the entropy change of achieving the transition state (Adamson, 1973) and could reflect physical limitations on the reaction, such as those produced by hydrogen-bonding, base-stacking interactions, and the covalent bonds that comprise the sugar–phosphate backbone. These interactions constrain the cytosine hydrate in the double-stranded copolymer and reduce the probability that the decay reaction will take place, as compared with the decay (reversal) of the monomeric cytosine hydrate in solution. Additional steric and electronic factors may also be subsumed by the independent term of the Arrhenius equation.

The activation energy for the decay of cytosine hydrate within the copolymer as determined in these experiments was similar to the activation energies for the decay of hydrates of cytosine (21 kcal/mol), cytidine (13 kcal/mol), 3′-CMP (15.5 kcal/mol), and CMP (16 kcal/mol) in solution (Fisher & Johns, 1976). This indicates that the activation state for the reaction is stabilized to a degree similar to that of the starting material for the reaction, independent of whether the reaction occurs within a copolymer or occurs free in solution.

Hydrates of cytosine, deoxycytidine, and deoxycytidine monophosphate monomers in solution decay primarily by dehydration. Deamination typically accounts for less than 10% of the decay of cytosine hydrates (Fisher & Johns, 1976). In poly(dG-dC), cytosine hydrate also appears to decay primarily by dehydration to cytosine. Our results also suggest that some uracil hydrate is formed from cytosine in irradiated poly(dG-

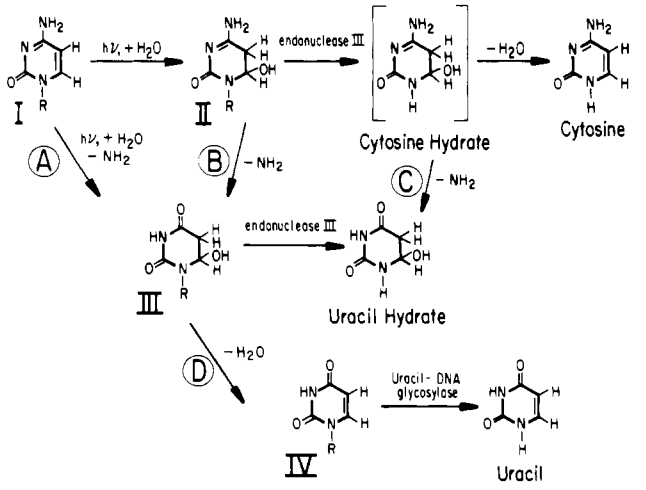

FIGURE 4: Outline of proposed mechanisms for formation and repair of cytosine photoproducts in DNA: compound I, cytosine in DNA; compound II, cytosine hydrate in DNA; compound III, uracil hydrate in DNA; compound IV, uracil in DNA.

dC). We have previously demonstrated that uracil hydrate formed by irradiation of poly(dA-dU) is a substrate for endonuclease III (Boorstein et al., 1989). We show here that uracil hydrate is also recovered following incubation of irradiated poly(dG-dC) with endonuclease III.

We have outlined in Figure 4 three possible mechanisms (reactions A–C) by which the uracil hydrate might have been formed. In reactions A and B, uracil hydrate is formed in the copolymer. In reaction C, uracil hydrate results from the deamination of cytosine hydrate after enzymatic release from the copolymer. This last reaction would be an artifact of the assay system and would not be of biological significance. Evidence that uracil hydrate was, in fact, formed from cytosine in poly(dG-dC) is the observation that, at time "0", no uracil was released from irradiated poly(dG-dC) incubated with uracil–DNA glycosylase. However, incubation of poly(dG-dC) with uracil–DNA glycosylase 24 h after irradiation resulted in release of uracil in an amount equal to 1.5% of the total of the time 0 enzymatically released photohydrates. This uracil could only have been formed via the dehydration of uracil hydrate in the copolymer following UV irradiation. Therefore, the contribution of artifactual reaction C to the formation of uracil hydrate was probably not significant.

Reaction B is identical with reaction C except that it occurs on the DNA backbone and is therefore of great biologic significance. These two reactions describe deamination resulting from the instability of the bond linking the exocyclic amino group to the hydrate pyrimidine ring (Kochetkov & Budovskii, 1971). At time 0, after 60 min of irradiation at 0 °C and 30 min of incubation with endonuclease III at 37 °C, approximately 4% of the released photohydrates were uracil hydrate. Had this uracil hydrate been formed exclusively via reaction B, then by 24 h after irradiation most of the cytosine hydrate should have been deaminated and released as uracil hydrate or uracil. However, the maximum yield of enzymatically released uracil hydrate and uracil together never exceeded 8–10% of that released as cytosine. We are forced to conclude that a significant percentage of the uracil hydrate formed resulted from reaction A which occurred during the half hour of irradiation at 0 °C. It is not certain whether reaction A is a concerted photochemical reaction or whether it represents a series of discrete steps. Its biologic significance is also not certain since the UV dose was quite high and, of necessity, was administered over a relatively long time period. Nevertheless, these results unambiguously show that UV irradiation of cytosine residues in DNA results in significant deamination.

In turn, uracil hydrate in poly(dA-dU) or in poly(dG-dC) decays by dehydration to uracil (Figure 4, reaction D). The dehydration of uracil hydrate in poly(dA-dU) had a higher activation energy than did the dehydration of cytosine hydrate in poly(dG-dC). Uracil hydrate in solution also dehydrates with a higher activation energy than does cytosine hydrate (Fisher & Johns, 1976). However, as compared with the hydrate of cytosine, uracil hydrate in the copolymers was not stabilized to the same extent as was cytosine hydrate. We found that uracil hydrate in poly(dA-dU) was stable at 25 °C and had a half-life of 6 h at 37 °C and of less than 0.5 h at 55 °C. These half-lives are similar to those that we have calculated from reaction constants reported in Fisher and Johns (1976). Uracil hydrate in its monomeric form reverted at pH 7.0 with half-lives of 107 h at 20 °C, 79 h at 27 °C, and 6.8 and 4.4 h in two different determinations at 50 °C. This lack of stabilization in the copolymer might be because uracil hydrate is only capable of making two high hydrogen bonds, as compared with cytosine hydrate, which might form three. Regardless, our results demonstrate that uracil can be a product of UV irradiation of cytosine via a uracil hydrate intermediate. Uracil formed in this manner would be located opposite guanine and would therefore be mutagenic.

Previously, Lecomte et al. (1981) postulated the existence of three nondimeric photoproducts in UV-irradiated poly(dC) by studying the effect of irradiation on the coding and template properties of this polymer. They detected a short-lived reversible photoproduct that coded for cytosine and, to a lesser extent, thymine. They inferred that this photoproduct was cytosine hydrate. A second base modification, which coded for adenine and was uracil–DNA glycosylase resistant, was considered to be uracil hydrate. Finally, the adenine-coding lesions were lost by heating and uracil–DNA glycosylase treatment, consistent with the formation of uracil from uracil hydrate. Our results differ from theirs in one respect. The lesion they identify as cytosine hydrate was more labile than was cytosine hydrate in our experiments. However, this discrepancy is readily reconciled, since their experiments were in single-stranded polymers, while ours were in a double-stranded copolymer. Hydrogen bonding in the latter might account for the additional stability.

Vanderhoek and Cerutti (1973) used a degradative assay to measure the cytosine photohydrate content of DNA. With this assay they obtained results significantly different from ours. This discrepancy is probably the result of their use of a 25-h incubation at a high alkaline pH (10.3) to effect borohydride reduction. It is probable that this incubation resulted in significant denaturation of the native DNA during which time the photohydrates reverted to cytosine rather than undergoing reduction. This might have resulted in an underestimation of the cytosine hydrate content, and thus an underestimation of the half-life.

Taken together, the results of these experiments indicate that the cytosine hydrate moiety is stabilized when formed in DNA as compared to its formation in cytosine, deoxycytidine, and deoxycytidine monophosphate. A small but significant percentage of cytosine hydrate moieties in DNA undergo deamination to uracil hydrate and then eliminate water, yielding uracil. This confirms that the reaction sequence which has been shown to yield uracil after UV irradiation of cytosine in solution also occurs in DNA (Shapiro, 1981). Since the replication of these lesions may lead to transitions and transversions, it is probable that their formation in DNA promoted the evolutionary development of endonuclease III and the

analogous enzymes of higher organisms, together with uracil–DNA glycosylase.

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Registry No. Poly(dG-dC), 36786-90-0; poly(dA-dU), 34607-75-5; poly(dG-[^{3}H]dC), 121124-87-6; poly(dA-[^{3}H]dU), 121124-89-8; uracil, 66-22-8; cytosine hydrate, 10010-80-7; uracil hydrate, 1194-23-6; endonuclease III, 60184-90-9.

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