Monomeric Base Damage Products from Adenine, Guanine, and Thymine Induced by Exposure of DNA to Ultraviolet Radiation†

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ABSTRACT: The formation of monomeric products in DNA upon exposure to UV radiation was investigated. Three novel products were identified in DNA in aqueous solution upon exposure to UV radiation at 254 nm in a dose range from 100 to 10 000 J/m^2. These were 4,6-diamino-5-formamidopyrimidine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and 5-hydroxy-5,6-dihydrothymine. These three products are known to be substrates for base excision repair enzymes involved in the reversal of oxidative DNA damage. The dependence of the yields of formamidopyrimidines on UV radiation dose was nonlinear, whereas the yield of 5-hydroxy-5,6-dihydrothymine was increased linearly in the entire dose range. Of these products, 4,6-diamino-5-formamidopyrimidine was the only compound produced in appreciable amounts at 310 nm. At the highest dose used, the formation of other pyrimidine- and purine-derived products was also observed. Their amounts, however, were increased above control levels up to 2-fold only. The hydroxyl radical scavenger dimethyl sulfoxide had no effect on product yields excluding the involvement of hydroxyl radical in product formation. 4,6-Diamino-5-formamidopyrimidine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine may be produced by hydration of adenine and guanine, respectively, across the N(7)−C(8) double bond by mechanisms similar to those proposed previously for well-known formation of pyrimidine hydrates with the hydroxyl group located at C(6). Formation of 5-hydroxy-5,6-dihydrothymine indicates that hydration of thymine with the hydroxyl group located at C(5) of the pyrimidine ring also occurs. The results of these studies indicate that these monomeric base damage products could be a biologically important component of the photoproducts that are responsible for the deleterious effects of UV radiation.

Ultraviolet (UV)[1] radiation plays a major role in the development of skin cancers (Engel et al., 1988). The main biological effects of UV radiation result from its interaction with DNA in cells. Exposure of DNA to UV radiation generates a number of products. The major photoproducts are cyclobutane pyrimidine dimers (CPDs) consisting of thymine−thymine dimers as major products with smaller amounts of cytosine−thymine dimers and cytosine−cytosine dimers [for reviews, see Fisher and Johns (1976a) and Cadet and Vigny (1990)]. Other dimeric products, which are involved in biological effects of UV radiation, are pyrimidine (6−4) pyrimidone adducts and the so-called spore photoproducts 5,6-dihydro-5-(α-thyminyl)thymidines. UV-radiation-induced monomeric products of DNA are the so-called pyrimidine photohydrates that are generated by addition of a water molecule across the C(5)−C(6) double bond with the hydroxyl group located at C(6) [for reviews, see Fisher and Johns (1976b) and Cadet and Vigny (1990)]. Hydration of cytosine predominates the photohydration process with hydration of thymine being a minor process (Cadet & Vigny, 1990). UV-radiation-induced formation of analogous purine products and hydration of pyrimidines with the hydroxyl group located at C(5) in DNA have not been reported.

Although it is generally recognized that the deleterious biological effects of UV light can be attributed primarily to the major UV photoproducts, CPDs and (6−4) photoproducts, there is evidence that other, less frequently occurring photoproducts may also be mutagenic. Mutational analyses in both bacterial and mammalian systems indicate that a subset of the spectrum of UV light-induced mutations occurs at non-CPD and (6−4) photoproduct sites and are likely to involve monomeric sites of purines and pyrimidines (LeClerc et al., 1984; Schaaper et al., 1987; Romac et al., 1989). In addition, a number of studies have shown that endonucleases associated with both partially and highly purified DNA repair enzymes recognize and cleave UV-irradiated DNA at sites of monomeric photoproducts occurring at any of the four DNA bases. In some cases, these activities have been shown to be directed against the 6-hydroxy-5,6-dihydro derivatives

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[1] Abbreviations: UV, ultraviolet; CPD, cyclobutane pyrimidine dimer; DMSO, dimethyl sulfoxide; GC/MS-SIM, gas chromatography−mass spectrometry with selected-ion monitoring; 5-OH-5-MeHyd, 5-hydroxy-5-methylhydantoin; 5-OH-Hyd, 5-hydroxyhydantoin; 5-OH-5,6-diHThy, 5-hydroxy-5,6-dihydrothymine; 5-OHMeUra, 5-(hydroxymethyl)uracil; 5-OH-Ura, 5-hydroxyuracil; 5-OH-Cyt, 5-hydroxycytosine; 5,6-diOH-Ura; 5,6-dihydroxyuracil; Thy gly, thymine glycol; FapyAde, 4,6-diamino-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; 2-OH-Ade, 2-hydroxyadenine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Gua, 8-hydroxyguanine.

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of cytosine, uracil and thymine (Boorstein et al., 1989, 1990; Ganguly et al., 1990). However, the nature of the guanine and adenine photoproducts cleaved by these endonuclease activities remains unknown (Helland et al., 1986; Gossett et al., 1988; Gallagher & Duker, 1986 1989). Thus, the identification of UV-light-induced purine photoproducts is an important step toward the characterization of such DNA repair enzymes as well as in defining the role of these monomeric lesions in the deleterious biological effects of UV radiation.

In the present work, we have studied the formation of a number of monomeric products that can be generated in DNA upon UV irradiation. A number of these products are known to be generated by ionizing radiation and oxidizing agents and their association with UV radiation exposure of DNA was unexpected. The relevance of these products with respect to the biological effects of UV radiation is discussed from the standpoint of their recognition by two well-characterized types of DNA repair enzymes as well as their potential toxicity and mutagenicity.

EXPERIMENTAL PROCEDURES

Materials.[2] Modified DNA bases, their stable isotope-labeled analogues, thymine-α,α,α,*6*-d_4 and materials for gas chromatography/mass spectrometry (GC/MS) were obtained as described (Dizdaroglu, 1993, 1994).

Preparation of Plasmid DNA. Large-scale preparations of pGEM-2 (2869 bp) plasmid DNA were generated (Qiagen Megaplasmid kit) under conditions that minimized the introduction of oxidative damage (e.g., no exposure to phenol). Each plasmid preparation used was checked for oxidative DNA damage utilizing a plasmid nicking assay following treatment with *Escherichia coli* endonuclease III (Gossett et al., 1988). DNA was used within 1 week of preparation.

Irradiations. DNA samples were dissolved in 10 mM KH_2PO_4 at pH 7.5 (buffer K) at a concentration of 0.2 mg/mL, and 250 μL droplets were irradiated with either a short wavelength (254 nm), 15 W lamp (Photodyne) or a medium wavelength (calibrated at 310 nm), 15 W lamp (Photodyne) at room temperature. UV fluence was measured with a calibrated radiometer (UVP, model UVX). Following irradiation, samples were precipitated with ethanol and the pellets were washed with 70% ethanol, resuspended in 0.5 mL of buffer K, and reprecipitated. The pellets were lyophilized prior to further analysis. For experiments with dimethyl sulfoxide (DMSO), samples were treated as described above except the buffer contained 0.5 M DMSO.

Analysis by Gas Chromatography/Mass Spectrometry (GC/MS). Aliquots of thymine-α,α,α,*6*-d_4 and stable isotope-labeled analogues of modified DNA bases were added as internal standards to DNA samples containing 50 μg of DNA (Dizdaroglu, 1993, 1994). Samples were hydrolyzed with 60% formic acid for 30 min at 140 °C in vials (sealed under N_2) and then freeze-dried. Hydrolysates were derivatized with 100 μL of a mixture containing bis(trimethylsilyl)-trifluoroacetamide and acetonitrile (4:1, v/v) for 30 min at 120 °C in vials (sealed under N_2) and then analyzed by GC/MS with selected ion-monitoring (SIM) (Nackerdien et al., 1992). A mass spectrometer (Hewlett-Packard Model 5989A MS Engine) interfaced to a gas chromatograph (Hewlett-Packard Model 5890 Series II) equipped with an automatic injector was used for this purpose. The injection port of the gas chromatograph and the GC/MS interface were kept at 250 and 280 °C, respectively. The ion source was maintained at 280 °C. Other conditions of analysis were as described previously (Nackerdien et al., 1992). The quantification of modified DNA bases was done by isotope-dilution mass spectrometry (Dizdaroglu, 1993, 1994). The stable isotope-labeled analogue of 5-hydroxy-5,6-dihydrothymine (5-OH-5,6-diHThy) was not available. This compound was quantified using 6-azathymine as a standard (Fuciarelli et al., 1989; Nackerdien et al., 1992). Thymine-α,α,α,*6*-d_4 was used as an internal standard for thymine to verify the amount of DNA (Djuric et al., 1991).

RESULTS

The purpose of this work was to determine whether exposure of DNA in aqueous solution to UV radiation causes formation of monomeric products other than those previously identified. Aerated DNA solutions were exposed to UV radiation of 254 or 310 nm at doses from 50 to 10 000 J/m^2. Control and UV-irradiated DNA samples were analyzed by GC/MS-SIM for modified DNA bases. Eight pyrimidine-derived and five purine-derived lesions were identified and quantified. These were 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 5-hydroxyhydantoin (5-OH-Hyd), 5-hydroxy-5,6-dihydrothymine (5-OH-5,6-diHThy), 5-(hydroxymethyl)-uracil (5-OHMeUra), 5-hydroxyuracil (5-OH-Ura), 5-hydroxycytosine (5-OH-Cyt), 5,6-dihydroxyuracil (5,6-diOH-Ura), thymine glycol (Thy glycol), 4,6-diamino-5-formamidopyrimidine (FapyAde), 8-hydroxyadenine (8-OH-Ade), 2-hydroxyadenine (2-OH-Ade), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and 8-hydroxyguanine (8-OH-Gua). Except for 5-OH-5,6-diHThy, these modified bases were present in control DNA samples at detectable levels. In UV-irradiated DNA (254 nm), the amounts of most modified bases were increased significantly over control levels at the highest dose 10 000 J/m^2 only. Three modified bases FapyAde, FapyGua, and 5-OH-5,6-diHThy were the exceptions. Their formation was also observed at lower doses starting from 100 J/m^2.

Figure 1 illustrates the dose-yield plots of FapyAde in the dose range (254 nm) from 50 to 1000 J/m^2 and from 50 to 10 000 J/m^2. Significant increases over the control amount of FapyAde were observed at the dose range from 100 to 10 000 J/m^2. At 50 J/m^2, the lowest dose used in this study, no significant increase over the control level of FapyAde was detected. Up to 200 J/m^2, there was a dose-dependent linear increase in the yield of this product. Above 200 J/m^2, the yield deviated from linearity reaching a plateau between the doses 5000 and 10 000 J/m^2. Figures 2 and 3 represent the dose-yield plots for formation of FapyGua and 5-OH-5,6-diHThy, respectively. UV irradiation of DNA at 254 nm in the dose range from 500 to 10 000 J/m^2 resulted in significant increases in the amount of FapyGua (Figure 2). Similar to the formation of FapyAde above 200 J/m^2, the dependence of formation of this product on the radiation dose was nonlinear. 5-OH-5,6-diHThy was not detectable in

[2] Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

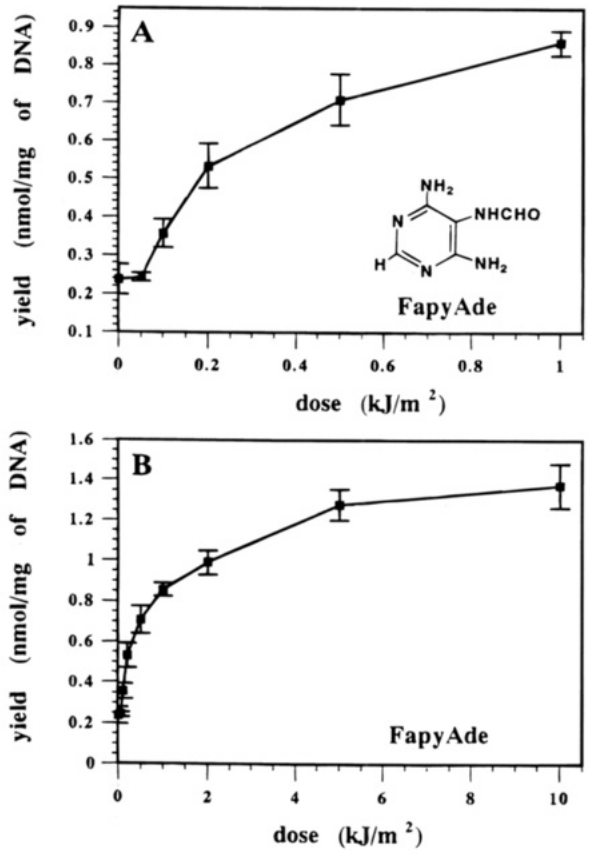

FIGURE 1: Dose-yield plot of 4,6-diamino-5-formamidopyrimidine (FapyAde). (A) In the range of 0−1 kJ/m^2 of UV irradiation at 254 nm. (B) In the range of 0−10 kJ/m^2 of UV irradiation at 254 nm. Each data point represents the mean of three values that were obtained by measurement of three independently prepared DNA samples. Error bars represent standard deviations. 1 nmol of a modified base/mg of DNA corresponds to ≈32 modified bases per 10^5 DNA bases.

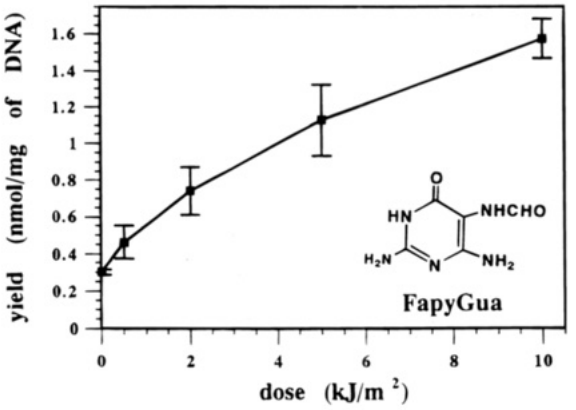

FIGURE 2: Dose-yield plot of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua). DNA samples were UV-irradiated (254 nm) at the indicated doses. Each data point represents the mean of three values that were obtained by measurement of three independently prepared DNA samples. Error bars represent standard deviations.

control DNA samples, and small quantities of this modified base were detected in DNA exposed to doses in the range from 500 to 10 000 J/m^2 (Figure 3). In contrast to FapyAde and FapyGua, a linear increase in the yield of 5-OH-5,6-diHThy over the entire dose range was observed. The yields of the other ten products at 10 000 J/m^2 and their control levels are illustrated in Figure 4A,B. The increases over control levels were no more than 2-fold. We conclude from

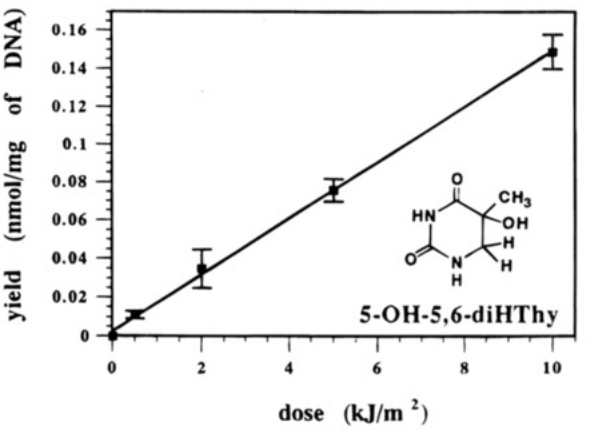

FIGURE 3: Dose-yield plot of 5-hydroxy-5,6-dihydrothymine (5-OH-5,6-diHThy). DNA samples were UV-irradiated (254 nm) at the indicated doses. Each data point represents the mean of three values that were obtained by measurement of three independently prepared DNA samples. Error bars represent standard deviations.

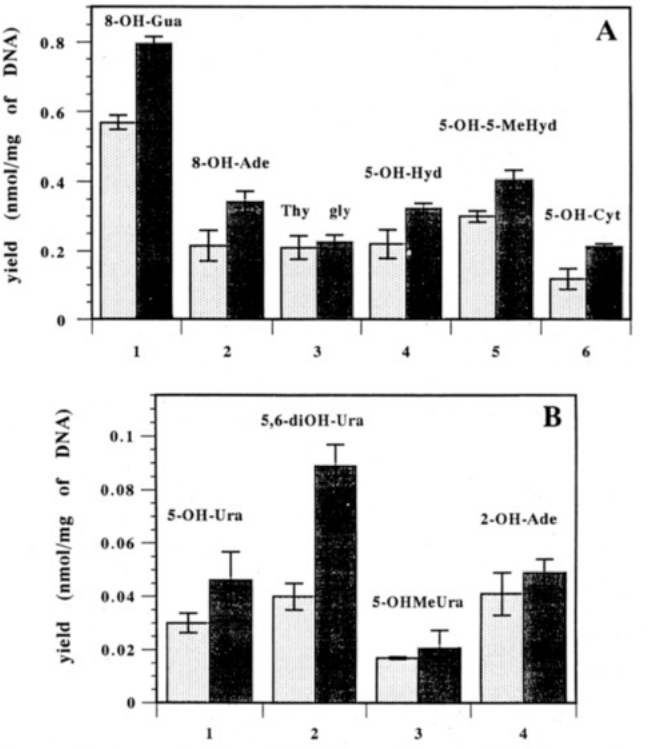

FIGURE 4: Amounts of modified bases in control DNA samples and in DNA samples exposed to 10 kJ/m^2 of UV irradiation at 254 nm. Light bars, control samples; dark bars, irradiated samples. (A) Yields of 8-OH-Gua (1), 8-OH-Ade (2), Thy gly (3), 5-OH-Hyd (4), 5-OH-5-MeHyd (5), and 5-OH-Cyt (6). (B) Yields of 5-OH-Ura (1), 5,6-diOH-Ura (2), 5-OHMeUra (3), and 2-OH-Ade (4). Each amount represents the mean of three values that were obtained by measurement of three independently prepared DNA samples. Error bars represent standard deviations.

these experiments that appreciable amounts of both FapyAde and FapyGua are generated from short wavelength (254 nm) UV irradiation of DNA over a range of low and high doses and that small amounts of a large variety of other monomeric base damage products are formed at high doses.

We wished to determine whether or not these photoproducts were also formed at longer UV wavelengths such as those associated with the UVB component (280−315 nm) of solar radiation. For these experiments, DNA samples were irradiated at 310 nm over a broad range of doses. FapyAde was the only modified base formed in appreciable amounts

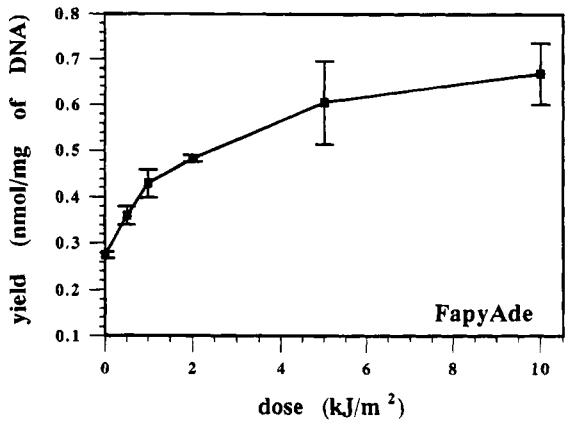

FIGURE 5: Dose-yield plot of 4,6-diamino-5-formamidopyrimidine (FapyAde) in the dose range of 0−10 kJ/m^2 of UV irradiation at 310 nm. Each data point represents the mean of three values that were obtained by measurement of three independently prepared DNA samples. Error bars represent standard deviations.

at this wavelength. The dose-yield plot for FapyAde formation at 310 nm is presented in Figure 5. There was a linear increase in the yield up to 1000 J/m^2 followed by a nonlinear increase. For a given UV dose, the overall yield for the formation of this photoproduct at 310 nm is approximately half that observed at 254 nm. Nevertheless, appreciable quantities of FapyAde are formed at UVB wavelengths.

Because FapyAde, FapyGua, and 5-OH-5,6-diHThy are formed in DNA in reactions mediated by hydroxyl radical attack [for a review, see Dizdaroglu (1992)], we wished to determine whether or not UV radiation might produce these monomeric base damages via a similar mechanism. The effect of a hydroxyl radical scavenger, dimethyl sulfoxide (DMSO), on product formation was tested. At a concentration of 0.5 M, DMSO had no effect on product yields (data not shown). When Tris buffer was used instead of phosphate buffer for DNA solutions, the yields of products were similar to those in phosphate buffer. Similar results were obtained for the other base damage products (data not shown). We conclude from these results that the formation of these novel monomeric base damage products by UV radiation does not involve participation of hydroxyl radicals.

DISCUSSION

The results of this study demonstrate for the first time that exposure of DNA in aqueous solution to far UV radiation at relatively low doses causes formation of three monomeric base damage products, namely, FapyAde, FapyGua, and 5-OH-5,6-diHThy. Of these products, FapyAde had the highest yield and appreciable amounts were observed at doses starting at 100 J/m^2. At the highest dose 10 000 J/m^2, the formation of a myriad of other pyrimidine- and purine-derived lesions were also observed. However, the amounts of some of these lesions were only slightly (although significantly) increased over control levels (Figure 4). All of the modified bases investigated in the present work are well known base damage products of DNA, which are produced by mechanisms involving free radicals, mainly hydroxyl radical upon exposure of DNA solutions to ionizing radiation [for reviews, see Téoule (1987) and Dizdaroglu (1992)]. In the present work, however, experiments performed with the use of DMSO clearly excluded the involvement of the hydroxyl radical in the mechanisms underlying formation of these products by UV irradiation. Moreover, product yields were not affected when Tris buffer was used instead of phosphate buffer. This supports the notion that hydroxyl radical was not involved in product formation since Tris can also act as a radical scavenger due to the high rate of its reaction with hydroxyl radical (Hicks & Gebicki, 1986).

In the past, bipyrimidine dimers [CPDs and (6−4) photoproducts] have been detected as major products of UV irradiation of nucleobases [for reviews, see Fisher and Johns (1976a,b) and Cadet and Vigny (1990)]. Our results indicate that approximately 15 FapyAde (Figure 1) and 5 FapyGua (Figure 2) residues are formed per 10^5 bases of plasmid DNA irradiated *in vitro* with 500 J/m^2 of UV radiation (254 nm). This compares with a CPD lesion frequency in UV-irradiated plasmids of about 385 per 10^5 bases for the same UV dose (Mitchell et al., 1991). The estimated frequency for 5-OH-5,6-diHThy (Figure 3) is about 40-fold lower than the purine photoproducts. Although these purine photoproducts occur at frequencies well below those for CPDs and (6−4) photoproducts, they occur at approximately the same frequency as cytosine photohydrates (Mitchell et al., 1991). Furthermore, at 310 nm, the ratio of FapyAde to CPDs is higher than it is at 254 nm. It is conceivable that FapyAde and FapyGua, if mutagenic, could account for some UV-induced mutations seen at non-bipyrimidine sites in both bacterial and mammalian systems (LeClerc et al., 1984; Schaaper et al., 1987; Romac et al., 1989). In addition, it is also possible that a subset of the UV-induced mutations in the human p53 gene observed in nonmelanoma skin cancers can be attributed to guanine and adenine photoproducts since either pyrimidine or purine precursors could be responsible for the observed base sequence changes (Ziegler et al., 1993; D. E. Brash, personal communication). Although there is currently no direct information regarding the mutagenicity of FapyGua or FapyAde, a closely related base damage product, 2,6-diamino-4-hydroxy-5-(N-methylformamido)pyrimidine is both a toxic and mildly mutagenic DNA lesion *in vivo* (Tudek et al., 1992) and arrests DNA polymerase *in vitro* (Boiteux & Laval, 1983; O'Connor et al., 1988). However, such extrapolations need to be interpreted with caution since slight structural changes can greatly alter the toxicity and mutagenicity of various base damage products (Ide et al., 1991; Maccabee et al., 1994).

A number of previous studies have reported the existence of activities present in prokaryotic DNA repair enzyme preparations that recognize and cleave UV-irradiated DNA at sites of both adenines (Gallagher & Duker, 1986, 1989; Doetsch et al., 1988) and guanines (Helland et al., 1986; Gossett et al., 1988). Both FapyGua and FapyAde are substrates for the *E. coli* Fpg protein (Breimer, 1984; Boiteux et al., 1992) and, presumably, a similar activity present in mammalian cells (Laval et al., 1990). Hence, the previously reported "G-cutter" and "A-cutter" activities on UV-damaged DNA can now be accounted for by the presence of Fpg or Fpg-like proteins cleaving DNA at sites of UV radiation-induced FapyGua and FapyAde. In addition, 5-OH-5,6-diHThy is a substrate for *E. coli* endonuclease III (Dizdaroglu et al., 1993) and presumably eukaryotic endonuclease III counterparts.

Pyrimidine hydrates have been found to be 6-hydroxy-5,6-dihydro type products of cytosine and uracil with photohydration of thymine being much less prominent (Fisher

& Johns, 1976a; Cadet & Vigny, 1990). The formation of 5-hydroxy-5,6-dihydro type products had been ruled out by those investigators. Mechanistic studies have indicated that a singlet excited state precedes photohydration (Burr et al., 1968, 1975; Wang & Nnadi, 1968; Summers & Burr, 1972). Mechanisms involving a zwitterionic intermediate and a pyrimidine carbocation have also been proposed (Wang et al., 1956; Khattak et al., 1972; Garner & Scholes, 1985). Mechanisms underlying formation of FapyAde and FapyGua may be analogous to those proposed for hydration of pyrimidines. Hydration of the imidazole ring of purines across the N(7)−C(8) double bond with the hydroxyl group located at C(8) may yield purine hydrates 7-hydro-8-hydroxyadenine and 7-hydro-8-hydroxyguanine. These compounds being hemiorthoamides are sensitive to hydrolysis and may yield formamidopyrimidines FapyAde and FapyGua (Steenken, 1989), respectively, which were identified as products of UV irradiation of DNA in the present work. On the other hand, studies with UV-irradiated frozen aqueous model systems provided evidence for formation of adenine and guanine radical cations as a result of photoionization with likely involvement of a triplet-state intermediate (Hélene et al., 1966; Sevilla et al., 1979; Graslund et al., 1979; Arce & Rivera, 1984; Cadet & Vigny, 1990). If radical cations of adenine and guanine are formed in DNA, their reaction with water by addition of OH^- may result in formation of C(8)-OH adduct radicals (Symons, 1987). The C(8)-OH adduct radicals of purines, which are also produced by addition of hydroxyl radical to C(8) of purines, are known to yield formamidopyrimidines and purine hydrates upon one-electron reduction and 8-hydroxypurines upon one-electron oxidation [for a review, see Steenken (1989)]. For this reason, the formation of FapyAde and FapyGua in DNA upon UV irradiation may be due to generation of purine radical cations. On the other hand, the absence of production of 8-hydroxypurines at low UV doses may be an evidence against this mechanism involving radical cations. This is because the formation of 8-hydroxypurines from C(8)-OH adduct radicals in DNA has been found to be as prominent as that of formamidopyrimidines in the presence of oxygen (Fuciarelli et al., 1990; Gajewski et al., 1990).

The formation of 5-OH-5,6-diHThy may be an indication for alternative hydration of pyrimidines with the hydroxyl group located at C(5) of the pyrimidine ring. Alternatively, the mechanism underlying the formation of this product may involve a pyrimidine carbocation as described for formation of well-known pyrimidine hydrates (Garner & Scholes, 1985). Whether a pyrimidine carbocation upon hydrolysis may give rise to 5-OH-5,6-diHThy remains to be determined.

The dose-dependent increase in the yield of FapyAde was linear up to 200 J/m^2 and subsequently deviated from linearity reaching a plateau at higher doses. The formation of FapyGua was also nonlinear. In contrast, the yield of 5-OH-5,6-diHThy increased linearly with the UV-radiation dose in the entire dose range used. The deviation from linearity in the yields of formamidopyrimidines may be due to their aromatic character. This means that these compounds may absorb UV radiation and subsequently convert into other products. However, 5-OH-5,6-diHThy being a saturated compound should not absorb UV light at the wavelengths used in this work and thus not convert into other products. Therefore, the yield of this compound is expected to linearly increase with UV-radiation dose.

In conclusion, the formation of three novel monomeric products was observed in DNA in aqueous solution upon exposure to UV radiation. The formation of formamidopyrimidines indicates that hydration of purines in addition to well-known hydration of pyrimidines occurs in UV-irradiated DNA. It appears that thymine is also susceptible to hydration across the C(5)−C(6) double bond, but with hydroxyl group located at C(5). Mechanisms proposed in the past for UV-radiation-induced hydration of pyrimidines may also hold for purines. Mechanistic aspects of formation of formamidopyrimidines and thymine hydration with the hydroxyl group located at C(5) need to be investigated further.

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