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Effect of Hydration on the Induction of Strand Breaks and Base Lesions in Plasmid DNA Films by γ-Radiation

Akinari Yokoya,[†] Siobhan M. T. Cunniffe,[‡] and Peter O'Neill*,[‡]

Contribution from SPring-8, Japan Atomic Energy Research Institute, Hyogo 679-5148, Japan, and MRC Radiation and Genome Stability Unit, Harwell, Didcot, Oxon OX11 0RD, U.K.

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Abstract: The yields of γ-radiation-induced single- and double-strand breaks (ssb's and dsb's) as well as base lesions, which are converted into detectable ssb by the base excision repair enzymes endonuclease III (Nth) and formamidopyrimidine-DNA glycosylase (Fpg), at 278 K have been measured as a function of the level of hydration of closed-circular plasmid DNA (pUC18) films. The yields of ssb and dsb increase slightly on increasing the level of hydration (Γ) from vacuum-dried DNA up to DNA containing 15 mol of water per mole of nucleotide. At higher levels of hydration ($15 < \Gamma < 35$), the yields are constant, indicating that $H_2O^{\bullet+}$ or diffusible hydroxyl radicals, if produced in the hydrated layer, do not contribute significantly to the induction of strand breaks. In contrast, the yields of base lesions, recognized by Nth and Fpg, increase with increasing hydration of the DNA over the range studied. The maximum ratios of the yields of base lesions to that of ssb are 1.7:1 and 1.4:1 for Nth- and Fpg-sensitive sites, respectively. The yields of additional dsb, revealed after enzymatic treatment, increase with increasing level of hydration of DNA. The maximum yield of these enzymatically induced dsb is almost the same as that for prompt, radiation-induced dsb's, indicating that certain types of enzymatically revealed, clustered DNA damage, e.g., two or more lesions closely located, one on each DNA strand, are induced in hydrated DNA by radiation. It is proposed that direct energy deposition in the hydration layer of DNA produces $H_2O^{\bullet+}$ and an electron, which react with DNA to produce mainly base lesions but not ssb. The nucleobases are oxidized by $H_2O^{\bullet+}$ in competition with its conversion to hydroxyl radicals, which if formed do not produce ssb's, presumably due to their scavenging by Tris present in the samples. This pathway plays an important role in the induction of base lesions and clustered DNA damage by direct energy deposition in hydrated DNA and is important in understanding the processes that lead to radiation degradation of DNA in cells or biological samples.

Introduction

Ionizing radiation induces a variety of damages in DNA by both direct and indirect effects.[1] In living cells, ~40−50% of the lesions induced in DNA by low linear energy-transfer radiation arise from direct energy deposition events (direct effects), many of which ionize the DNA.[2] The biological effects of ionizing radiation are thought to arise from the formation of clustered DNA damage, e.g., two or more lesions (base lesion, single-strand breaks (ssb's), abasic site) formed within about 10 base pairs separation by a single radiation track. Most mechanistic studies[1,3] to date have focused on the indirect effects of radiation by which DNA damage is induced by diffusible water radicals. These studies, using dilute, aqueous solutions containing DNA, have revealed that the hydroxyl radical ($OH^{\bullet}$) is the main water radical that induces single- and double-strand breaks (dsb's) in DNA, whereas both hydrated electrons and $OH^{\bullet}$ induce DNA base lesions (for reviews, see refs 4 and 5). Much less is known about the mechanisms of the direct effects of radiation, which lead to persistent DNA damage. Although a few studies have shown that strand breaks are induced by radiation at ambient temperatures,[6,7] even less is know about the induction of base lesions in DNA by direct effects. Since cellular DNA, which is mainly in the B-form, contains about 20 water molecules per nucleotide in the first and second layers of hydration,[8,9] the role of these water molecules should also be considered in the mechanisms of degradation of DNA by direct energy deposition events in DNA.[10] Knowledge of the processes resulting in radiation degradation of DNA by direct effects will greatly increase our understanding of the deleterious effects of ionizing radiation in cells. Further, it is becoming recognized that degradation of biological samples in structural

* To whom correspondence should be addressed. E-mail: p.oneill@har.mrc.ac.uk. Tel.: +44 1235834393. Fax: +44 1235834776.
[†] Japan Atomic Energy Research Institute.
[‡] MRC Radiation and Genome Stability Unit.

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biology is an important consideration[11,12] for the next generation of synchrotrons used in X-ray crystallography.

It is difficult to study the effects of direct energy deposition in DNA in a dilute, aqueous environment, due to the overwhelming yield of water radicals, even when using high concentrations of radical scavengers to reduce the mean diffusion distance of water radicals to that within the cellular environment.[13,14] To date, most studies on the induction of DNA damage by direct energy deposition at ambient temperatures have concentrated on strand breaks induced in "dry" or hydrated DNA. Ito and co-workers[6] reported that the yield of strand breaks induced in plasmid DNA [in a dry, humid (assumed 20 water molecules per nucleotide ($\Gamma = 20$)) or aqueous state] by γ-irradiation at 77 or 298 K significantly increases on hydration of the DNA, especially for irradiations at 298 K. Γ is defined as the number of water molecules per nucleotide. In crystalline DNA ($\Gamma \approx 7$) at 293 K, sugar radicals, generated by direct ionization of DNA, were proposed to be precursors to strand breakage,[7] the yields of which are similar to those reported previously.[6] Using 193-nm light, monophotonic ionization events occur predominantly at the nucleobases of DNA, when present in an aqueous environment at 293 K.[15] The subsequent charge migration processes result in localization of the radical centers in the nucleobases, generally guanine for electron loss centers, which have a low probability of inducing strand breakage.[15]

Swarts and co-workers[16] showed that the yields of unaltered nucleobases, released from γ-irradiated salmon sperm DNA under anoxia at 293 K, decreased upon increasing the level of hydration of the DNA. They proposed that two separate processes are involved in base release, namely charge transfer from ionized water radicals formed in the inner hydration layer ($\Gamma < 15$) and $OH^{\bullet}$ generated in the outer layer ($\Gamma > 15$). Subsequently, Swarts and co-workers[17] demonstrated that the overall yield of radiation-induced base damage, based on the total mass of the sample, under anoxia is essentially independent of the level of hydration of DNA up to $\Gamma = 15$, above which the damage yield decreases. The major products resulting from electron loss processes are oxidized guanines, 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (Fapy) and 7,8-dihydro-8-oxo-2′-deoxyguanine (8-oxoG). The yields of electron loss products significantly exceed those of products arising from electron gain processes, although artifacts of the analytical methods used to detect oxidized guanine products have been discussed.[18] From EPR studies[19,20] on γ-irradiated DNA at 77 K, the $OH^{\bullet}$ was not observed if DNA contained less than nine water molecules per nucleotide, since any water radical cations ($H_2O^{\bullet+}$) generated in these water molecules are thought to oxidize DNA. When $\Gamma > 9$, the yield of $OH^{\bullet}$ substantially increases with increasing level of hydration, approaching the yield of $OH^{\bullet}$ determined for pure ice. Evidence of hole transfer from the hydration layer to DNA was proposed from measurement of the total yields of radicals induced in oligodeoxynucleotide crystals, X-irradiated at 4 K.[21]

In this study, we have provided the first evidence that DNA lesions, and in particular biologically relevant clustered DNA damage, are induced through the direct effects of γ-radiation of plasmid DNA at 278 or 310 K at different levels of DNA hydration. The yields of base lesions and clustered DNA damage have been determined by postirradiation treatment of the DNA with enzymatic probes (base excision repair proteins endonuclease III (Nth) and formamidopyrimidine-DNA glycosylase (Fpg)), which excise and convert base lesions into readily detectable DNA strand breaks.

Materials and Methods

Plasmid DNA Preparation. Plasmid DNA (pUC18, 2686 base pairs) was obtained from an *E. coli* HB101 host and extracted using alkalilysis, followed by purification with double banding on cesium chloride−ethidium bromide gradients as described previously.[22] The plasmid, which is over 90% in the closed-circular form, was subsequently stored at −20 °C in TE buffer (10 mmol dm^{-3} Tris, 1 mmol dm^{-3} EDTA, pH 8.0) at a concentration of 2.2 mg/mL.

Humidity Control of the DNA Samples. The stock solution of DNA was diluted with TE buffer to give a final DNA concentration of 0.22 mg/mL. Five-microliter aliquots of this plasmid solution at 4 °C were spotted onto a Hostaphan base of a glass-walled irradiation dish and dried by blowing dry air (<10% humidity) for 30 min to avoid crystallization of the buffer solutes by a flash-freeze-drying procedure. If crystallization occurs in the sample, subsequent liquefaction of the crystals may occur by absorption of water vapor at ambient temperature during the procedure to control the humidity. After drying, a uniform film of DNA/buffer solutes of 3 mm diameter was formed on the Hostaphan base of the dish. The dish was then placed in a freeze-drying apparatus (Micro-Modulin, Edwards) for 1 h to remove any additional water molecules loosely bound to the DNA sample. Three plasmid DNA films were normally prepared on a single dish.

To investigate the influence of humidity upon the induction of radiation-induced DNA damage, it is a prerequisite that any DNA damage induced by the experimental procedure for a specified level of hydration is insignificant. Any DNA damage induced through handling is particularly critical in the determination of the yield of radiation-induced dsb's, since a dsb may arise from the presence of a radiation-induced ssb produced spatially close to a ssb, induced by handling, on the complementary strand. Humidity control, carried out at room temperature or in the absence of salts, results in significant amounts of damage (>30% loss of the closed-circular form), making quantification of the yield of radiation-induced dsb's difficult against this background.[23] To minimize degradation of the DNA, air-dried DNA samples on the Hostaphan base of the dish were placed into a plastic chamber at 5.7 °C, together with a plastic dish (3 cm diameter) into which 2 mL of a sodium hydroxide solution was placed to maintain an appropriate humidity condition. The concentrations of sodium hydroxide of 8.18, 6.56, 4.80, and 3.84 mol/dm^{-3} give relative humidities of 73, 83, 91, and 97%, respectively, in the chamber. The relative humidities

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at 25 °C, as reported by Stokes and Robinson,[24] are ~12% lower than those at 5.7 °C. The relative humidity was monitored by the sensor of a hygrometer (Hygrotest 6400, Testoterm Ltd.) inserted in the sealed chamber. The humidity control chamber was maintained at 5.7 ± 0.5 °C for >15 h to equilibrate the samples prior to γ-irradiation based on mass changes with time of calf thymus DNA at these levels of hydration.[23] Under these conditions of humidity control, <5% degradation of the closed-circular DNA occurs. At 97% humidity, the thickness of the samples, stained with propidium iodide (10 μg/mL in the DNA stock solution) for visualization, are ~5 μm (10 μm at the film rim), determined using a confocal microscope.

For vacuum-dried conditions, plasmid DNA on the Hostaphan base of a dish was irradiated with γ-rays immediately after the freeze-drying procedure. During irradiation, the sample chamber was kept at <15% humidity by filling the chamber with dry air. It was assumed that exposure to dry air does not significantly affect the level of hydration of DNA, since the irradiation time (maximum 67 min) is short compared with that needed to attain the required humidity (15 h).

***γ*-Irradiation of the DNA Samples.** Following establishment of the required humidity, the chamber was placed into a larger plastic chamber. The space between the outer and inner chambers was filled with cooling gel bags at 5.7 °C, to ensure uniform dose within the sample and to minimize temperature changes. The chamber was placed onto a 1-cm-thick Perspex Petri dish filled with cooled agarose gel (5.7 °C) and then irradiated under aerobic conditions with ^{60}Co γ-rays through the base of the Petri dish. The dose rate, determined using a Farmer type 2570 dosimeter with a type 2581 (0.6 cm^3) ionization chamber, was 92 Gy/min. The maximum irradiation time of 67 min delivers a dose of 6 kGy. The humidity in the sealed chamber containing DNA remained constant during the irradiation (within ±1.8%). Although the temperature rose from 5.7 to 12 °C during a 1-h irradiation, this temperature change does not significantly affect the relative humidity (<2% decrease at 80% relative humidity) and the yield of strand breaks, since incubation of the irradiated DNA samples at 37 °C for 30 min does not result in a significant increase in the yield of strand breaks (see below). After γ-ray irradiation, the sample was recovered with 10 μL of sterilized distilled water (4 °C) and stored at −20 °C (for maximum 4 h), prior to determination of the yield of strand breaks by agarose gel electrophoresis.

Detection of Enzyme-Sensitive DNA Damage. Purified proteins, Nth and Fpg, were generous gifts from Prof. Rick Wood and Dr. Roldan-Arjona (Imperial Cancer Research Fund). Stock solutions (50% glycerol, 100 mmol dm^{-3} potassium phosphate, 0.1 mol dm^{-3} diethioethanol (DTT), and 0.005% Triton X-100)[25] of either Nth or Fpg at concentrations of 1.3 and 21 ng/μL, respectively, at pH 6.6 were stored at −20 °C.

Five microliters of sodium acetate (1 mmol dm^{-3}) and 30 μL of chilled ethanol were added to two of the three irradiated plasmid DNA samples and the control unirradiated samples, which had been stored at −20 °C after recovery from the Hostaphan dish. The solutions were left at −20 °C for 30 min to precipitate the DNA and subsequently centrifuged (Hermle Z323) at 13 000 rpm for 30 min at 4 °C. After decanting the liquid, the DNA pellets were rinsed with 70% ethanol. Following centrifugation, the resulting DNA pellet was dried for 10 min to remove any excess ethanol using an evaporator (Jouan RC10.10) connected to a cold trap (Jouan RCT90) and a rotary pump (Javac DD75). The plasmid DNA was disolved in 20 μL of reaction buffer (0.5 mmol dm^{-3} EDTA, 0.1 mol dm^{-3} KCl, 40 mmol dm^{-3} HEPES, 0.5 mmol dm^{-3} DTT, and 0.2 mg/mL bovine serum albumin (BSA)[15] at pH 8.0, and an aliquot of the enzyme was then added to each irradiated sample or a nonirradiated sample as control. The optimal incubation time and temperature for the Nth reaction (30 min at 37 °C), reported by Fulford,[23] were adopted on the basis of the following titration experiments undertaken in this study. The optimum concentrations of Nth (1.1 ng per 1 μg of DNA) and Fpg (19 ng/μL of DNA) were determined by treatment of DNA samples irradiated with 4 kGy or unirradiated DNA samples, with varying quantities of Nth (0.26−6.5 ng/μL) or Fpg (1−63 ng/μg DNA), respectively, for 30 min at 37 °C. The optimum conditions chosen for both Nth and Fpg do not cause degradation of the closed-circular form of the plasmid DNA. The optimal incubation time with Fpg was determined to be 30 min, since incubation of nonirradiated plasmid DNA with Fpg (19 ng/μg DNA) for longer times causes some degradation of the closed-circular form of DNA. The additive effect of incubation of irradiated DNA (equilibrated at 97% humidity) with both enzymes at 37 °C for 30 min was determined by addition of 1 μL of both Nth and Fpg stock solutions to 18 μL of the reaction buffer. Following incubation of irradiated DNA or controls with the enzymes, 5 μL of 0.5 mol dm^{-3} EDTA was added to quench the activity of the enzymes. The DNA solutions were then placed on ice prior to quantification by agarose gel electrophoresis.

The samples were categorized into three groups depending upon the postirradiation treatment. The first group was maintained at −20 °C (termed prompt ssb or prompt dsb in the remainder of this paper), the second was incubated at 37 °C for 30 min in the absence of enzyme (prompt + heat-labile ssb), and the third was incubated at 37 °C for 30 min in the presence of Nth, Fpg, or Nth+Fpg ("Nth+ssb", "Fpg+ssb", or "Nth+Fpg+ssb").

Quantification of the Yields of Strand Breaks in Irradiated Plasmid DNA. Prior to agarose gel electrophoresis, 5 μL of the loading buffer (0.1% bromophenol blue, 30% sucrose in TBE (89 mmol dm^{-3} Tris, 89 mmol dm^{-3} boric acid, 2 mmol dm^{-3} EDTA)) was added to the solutions containing irradiated or control DNA, which had been treated with or without the enzymes. Either 15 μL of solutions containing DNA treated with enzyme(s) or 7.5 μL of non-enzymatically treated solutions were placed into the well of a 1% agarose (Sigma Type 1-A) gel in TBE buffer at pH 7.1. The samples were run at typically 75 mV cm^{-1}, 7 mA, for 17 h at 5.7 °C. Following electrophoresis, the gel was stained with 30 μL of ethidium bromide (10 mg/mL) in 600 mL of TBE buffer for 1 h at 5.7 °C. The separated closed-circular, open-circular, and linear forms of the plasmid DNA in the gel were visualized using a UV transilluminator, and an image of the gel was obtained using a charge-coupled device (CCD) camera. The relative amount of DNA in each form was then quantified as described previously.[22] The effect of superhelical density on the correction factor for ethidium binding to closed-circular DNA has been discussed previously.[22,26]

A dose−response was determined from the logarithmic loss of closed-circular plasmid DNA on radiation dose at the specified humidity. From the slope of this response, a D_{37} value was obtained which, assuming Poisson statistics for ssb induction, represents the radiation dose required to give on average one ssb per plasmid molecule. Using the D_{37} value, an average number of ssb/Gy/Da (n(ssb)) was obtained, assuming an average mass of a base pair of 650 Da and knowing that pUC18 DNA contains 2686 base pairs.

$$n(\text{ssb}) = 1/(2686 \times 650 \times D_{37}) \quad (1)$$

The average number of dsb/Gy/Da (n(dsb)) was determined from the dose dependence of the fractional abundance of the linear form of the DNA, given by

$$n(\text{dsb}) = b/(2686 \times 650) \quad (2)$$

where b is obtained from the slope of the dose−response.

***G* Values for Damage.** Careful consideration is needed to calculate radiation chemical yields (G values) for strand breaks in these hydrated DNA samples. For the dry/hydrated samples, it is reasonable to assume that a given γ-photon flux delivers the same dose to DNA molecules

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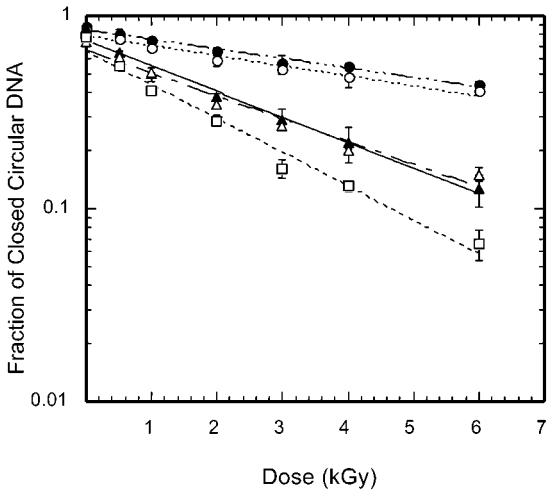

Figure 1. Dependence of the loss of closed-circular DNA on radiation dose after exposure of pUC18 plasmid DNA to ^{60}Co γ-rays at 5.7 °C and a relative humidity of 97% (○) or following a postirradiation incubated for 30 min at 37 °C in the absence (●) or presence of either Nth (▲), Fpg (△), or both Nth and Fpg (□). The points represent the mean ± SD of three independent experiments. Each line represents a least-squares fit to the data points.

for a fixed amount of DNA at the different levels of hydration. Based on this assumption, the *G* value (mol/J) is given by

$$G(\text{ssb}) = (1000/\text{mwt})/D_{37} \quad (3)$$

where mwt is the molecular weight of pUC18 plasmid DNA (1.746 MDa).

Results

Dependence of the Yield of ssb on the Level of Hydration. The dependence of the amount of closed-circular DNA on radiation dose is shown in Figure 1 for γ-irradiation of pUC18 plasmid DNA at 5.7 °C and at a relative humidity of 97%.

From this dependence, the yield of ssb was calculated from the D_{37} value (see eq 1). The yields of ssb were determined at different relative humidities from similar dose dependences for loss of closed-circular DNA irradiated at 5.7 °C. The dependence of the yield of ssb/Gy/Da on the level of hydration (Γ) is shown in Figure 2.

The mean value of Γ with standard deviation (see Table 1) was estimated from the relationship between relative humidity and the level of hydration of DNA reported in refs 16, 27−29. It has been assumed that Γ of vacuum-dried samples is $\sim 4 \pm 1.5$ under the conditions of the freeze-dry procedure. In previous studies, it was assumed that DNA contains ~2.5 water molecules per nucleotide under high vacuum[9] and ~5 water molecules per nucleotide at a low relative humidity (~20%).[30] The yield of prompt ssb slightly increases with increasing level of hydration when $\Gamma < 15$. At $\Gamma > 15$, the yield of 7×10^{-11} ssb/Gy/Da is independent of Γ. The yields of prompt ssb at various relative humidities are compiled in Table 1, together with the yields of prompt dsb (see below). Significant yields of additional ssb are not induced by a heat treatment, since the dependence of the yield of ssb/Gy/Da on Γ determined at

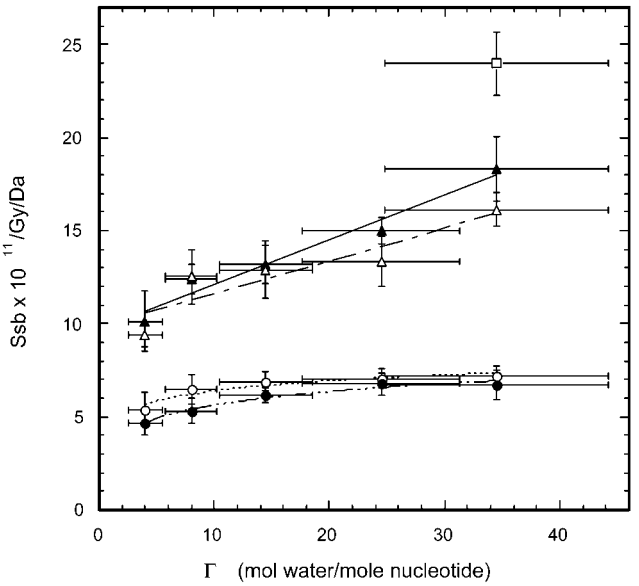

Figure 2. Dependence of the yield of ssb on Γ (moles of water molecule per nucleotide) for DNA samples irradiated at 5.7 °C (○) or following a postirradiation incubated at 37 °C for 30 min in the absence (●) or presence of either Nth (▲), Fpg (△), or both Nth and Fpg (□). The vertical error bars are ±SD of the slope of the dose−response curve for three independent experiments. The horizontal bars represent the mean values of Γ with standard deviation (see Table 1) estimated from the relationship between relative humidity and the level of hydration of DNA in refs 16, 27−29.

5.7 °C is similar to that determined after postirradiation heat treatment at 37 °C for 30 min (Figure 1).

Yields of Enzyme-Sensitive Sites Revealed by Nth and Fpg and Detected as ssb. Postirradiation incubation of the plasmid DNA with either Nth or Fpg at 37 °C results in a greater loss of closed-circular DNA than seen in the absence of an enzyme treatment for a given radiation dose, as shown in Figure 1. The yields of both Nth+ssb and Fpg+ssb, determined from dose dependences for the loss of closed-circular DNA, increase with increasing level of hydration, as shown in Figure 2 and Table 1. Taking the yields of Nth+ssb and Fpg+ssb to be 1.8×10^{-10} and 1.6×10^{-10} ssb/Gy/Da, respectively, at $\Gamma = 34.5$, the net yield of enzyme-sensitive sites, *n*(ess), may be obtained using eq 4.

$$n(\text{ess}) = n(\text{Nth (or Fpg)} + \text{ssb}) - n(\text{prompt} + \text{heat-labile ssb}) \quad (4)$$

taking *n*(prompt + heat-labile ssb) = 6.7×10^{-11} ssb/Gy/Da. The value of $n(\text{ess})_{\text{Nth+Fpg}}$ induced in irradiated DNA at $\Gamma = 34.5$ following incubation with both enzymes was estimated, assuming that the enzyme-sensitive sites (ess) recognized by both enzymes in irradiated plasmid DNA are independent.

$$n(\text{ess})_{\text{Nth+Fpg}} = n(\text{ess})_{\text{Nth}} + n(\text{ess})_{\text{Fpg}}$$

where $n(\text{ess})_{\text{Nth}}$ and $n(\text{ess})_{\text{Fpg}}$ are the yields listed in Table 2. The experimentally determined yield of ess induced in irradiated DNA following incubation with both enzymes is 1.7×10^{-10} ssb/Gy/Da. This value is ~80% of the estimated yield of $n(\text{ess})_{\text{Nth+Fpg}}$ of 2.1×10^{-10} ssb/Gy/Da, indicating that cross reactivity of Nth and Fpg for enzyme-sensitive sites, revealed as ssb's, is low. The values of *n*(ess) and the ratio of *n*(ess)/*n*(ssb) determined at $\Gamma = 34.5$ are compared in Table 2 with

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Table 1. Yields of ssb and dsb Induced in Dry pUC18 Plasmid DNA by γ-Irradiation under Different Levels of Hydration (with the Errors Shown in Parentheses)

treatment of sample	Γ (mol of water/ mol of nucleotide)	D_{37} $\times 10^3$ Gy	ssb/Gy/Da ($\times 10^{-11}$)	G(ssb) $\times 10^{-6}$ mol/J[b]	dsb/Gy/Da ($\times 10^{-11}$)	ssb/dsb
prompt damage	4 (1.5)	10.59	5.4 (0.9)	0.054 (0.009)	0.53 (0.05)	10
	8 (2.2)	8.83	6.5 (0.8)	0.065 (0.008)	0.54 (0.03)	12
	14.5 (4.1)	8.29	6.9 (0.5)	0.069 (0.005)	0.54 (0.11)	13
	24.5 (6.9)	8.10	7.1 (0.6)	0.071 (0.006)	0.58 (0.05)	12
	34.5 (9.7)	7.98	7.2 (0.5)	0.072 (0.005)	0.72 (0.07)	10
prompt + heat-labile damage (II)	4 (1.5)	12.32	4.7 (0.7)	0.047 (0.007)	0.43 (0.07)	11
	8 (2.2)	10.75	5.3 (0.7)	0.053 (0.007)	0.63 (0.14)	8
	14.5 (4.1)	9.30	6.2 (0.4)	0.062 (0.004)	0.74 (0.20)	8
	24.5 (6.9)	8.46	6.8 (0.6)	0.068 (0.006)	0.65 (0.04)	10
	34.5 (9.7)	8.54	6.7 (0.8)	0.067 (0.008)	0.72 (0.07)	9
II + Nth	4 (1.5)	5.67	10.1 (1.6)	0.10 (0.016)	0.69 (0.05)	15
	8 (2.2)	4.62	12.4 (0.8)	0.12 (0.008)	1.06 (0.18)	12
	14.5 (4.1)	4.34	13.2 (1.0)	0.13 (0.010)	1.23 (0.17)	11
	24.5 (6.9)	3.82	15.0 (0.7)	0.15 (0.007)	1.37 (0.13)	11
	34.5 (9.7)	3.13	18.3 (1.7)	0.18 (0.017)	1.71 (0.37)	11
II + Fpg	4 (1.5)	6.08	9.4 (0.6)	0.094 (0.006)	1.03 (0.18)	9
	8 (2.2)	4.58	12.5 (1.5)	0.13 (0.015)	1.46 (0.24)	9
	14.5 (4.1)	4.44	12.9 (1.6)	0.13 (0.016)	1.48 (0.21)	9
	24.5 (6.9)	4.31	13.3 (1.4)	0.13 (0.014)	1.83 (0.06)	7
	34.5 (9.7)	3.56	16.1 (0.9)	0.16 (0.009)	1.93 (0.06)	8
II + Fpg & Nth	34.5 (9.7)	2.39	24.0 (1.7)	0.24 (0.017)	2.37 (0.10)	10
high-scavenger solution[a]						
scavenging capacity 0.3×10^9 s^{-1}		1.02	56.0		1.6	35
scavenging capacity 1.4×10^9 s^{-1}		2.6	22.0		0.7	31

[a] Reference 23. [b] G values are based on the mass of DNA as described in the Materials and Methods section.

Table 2. Yields of Base Lesions Induced in pUC18 Plasmid DNA by γ-Irradiation at 5.7 °C and Γ = 34.5, Followed by Incubation with Nth and Fpg

treatment of sample	ess/Gy/Da $\times 10^{-11}$	n(ess)/ n(ssb)	$(ess)_{dsb}$/Gy/Da $\times 10^{-11}$	$(ess)_{dsb}$/ dsb
Nth	11.6	1.7	1.0	1.4
Fpg	9.4	1.4	1.2	1.6
Nth+Fpg	17.3	2.6	1.7	2.3
high scavenger solution[a]				
Nth	60.7 ± 16.3		2.76 ± 0.74	
Fpg	46.0 ± 11.9		2.09 ± 0.54	

[a] Reference 23. Scavenging capacity 1.4×10^9 s^{-1}.

data obtained from γ-irradiation of DNA in aqueous solution in the presence of high concentrations of the water radical scavenger Tris at 4 °C.[23]

Dependence of the Yield of dsb on the Level of Hydration. The induction of dsb by γ-irradiation of plasmid DNA at 5.7 °C or following incubation in buffer for 30 min at 37 °C increases linearly with increasing dose, as shown in Figure 3 for a relative humidity of 97% (Γ = 34.5). The yields of dsb/Gy/Da were calculated from the dose dependences using eq 2. Significant levels of additional dsb are not induced by heat treatment, since the yields of prompt dsb and prompt + heat-labile dsb at the various levels of hydration are similar, as shown in Figure 4. A slight increase with increasing value of Γ is seen in the lower hydration region (Γ < 15), but at higher values of Γ the yield of dsb/Gy/Da is constant. The maximum yield of prompt dsb at Γ = 34.5 is $\sim 7 \times 10^{-12}$ dsb/Gy/Da. The yields of dsb, n(dsb), at various levels of hydration are listed in Table 1, together with the ratios of n(ssb)/n(dsb).

Yield of Addition dsbs Revealed by Treatment with Nth and Fpg. Postirradiation incubation of the plasmid DNA with either Nth or Fpg at 37 °C results in the formation of a greater amount of linear DNA than that seen in the absence of an enzyme treatment at 97% relative humidity, as shown in Figure 3. The yields of both Nth+dsb and Fpg+dsb increase linearly

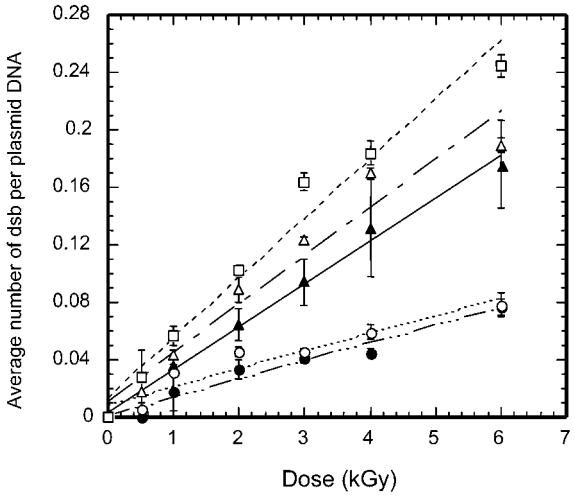

Figure 3. Dependence of the number of dsb's, determined from the fraction of the linear form of DNA, on radiation dose after exposure of pUC18 plasmid DNA to ^{60}Co γ-rays at 5.7 °C and a relative humidity of 97% (○) or following a postirradiation incubated for 30 min at 37 °C in the absence (●) or presence of either Nth (▲), Fpg (△), or both Nth and Fpg (□). The points represent the mean ± SD of three independent experiments. Each line represents a least-squares fit to the data points.

with radiation dose for all levels of hydration studied. The yields of both Nth+dsb and Fpg+dsb, indicative of clustered DNA damage, increase with increasing level of hydration, as shown in Figure 4. The yields of Nth+dsb are ~70−85% of those for Fpg+dsb at all levels of hydration studied. The maximum yields of Nth+dsb and Fpg+dsb at Γ = 34.5 are 1.7×10^{-11} and 1.9×10^{-11} dsb/Gy/Da respectively, comparable with a yield of 2.4×10^{-11} dsb/Gy/Da for treatment of irradiated DNA with both enzymes. At Γ = 34.5, the yields of dsb following enzyme treatment are 2−3 times larger than those for prompt dsb and prompt + heat-labile dsb. The ratios of ssb/dsb, however, do not depend significantly upon the level of hydration, as shown in Table 1. The yields of enzyme-sensitive sites detected as

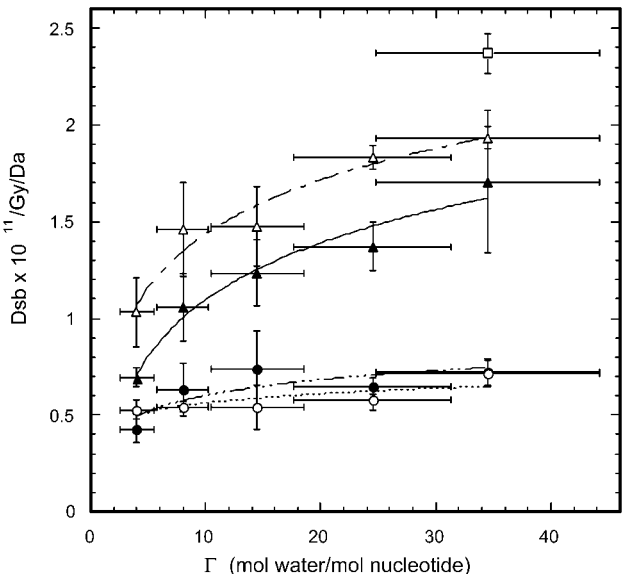

Figure 4. Dependence of the yield of dsb on Γ (moles of water molecule per nucleotide) for DNA samples irradiated at 5.7 °C (○) or following a postirradiation incubated at 37 °C for 30 min in the absence (●) or presence of either Nth (▲), Fpg (△), or both Nth and Fpg (□). The vertical error bars are ±SD of the slope of the dose−response curve for three independent experiments. The horizontal bars represent the mean values of Γ with standard deviation (see Table 1) estimated from the relationship between relative humidity and the level of hydration of DNA in refs 16, 27−29.

additional dsbs, $n(\text{ess})_{\text{dsb}}$, are given by

$$n_{\text{Nth(or Fpg)}}(\text{ess})_{\text{dsb}} = n(\text{Nth (or Fpg)} + \text{dsb}) - n(\text{prompt} + \text{heat-labile dsb}) \quad (5)$$

taking $n(\text{prompt} + \text{heat-labile dsb}) = 7.2 \times 10^{-12}$ dsb/Gy/Da. The values of $n(\text{ess})_{\text{dsb}}$ obtained at $\Gamma = 34.5$ are shown in Table 2.

Discussion

Control of the relative humidity of the plasmid DNA sample has made it possible to study systematically the effect of hydration on the induction of damage by direct energy deposition in the DNA−water complex, using enzymatic approaches to reveal base lesions. In the inner hydration layer ($\Gamma < 9$), water molecules are tightly associated with oxygen atoms of the phosphate group and sugar moiety (O(3′), O(5′)) and the heteroatoms of the bases.[8] In the outer hydration layer, the water molecules are loosely bound to DNA. The main findings from this study on the direct effects of radiation on hydrated DNA are the following: (i) the yields of radiation-induced prompt ssb are not significantly dependent upon the level of hydration of DNA compared with the large influence of hydration on the yield of base lesions, visualized as ess; (ii) heat-labile sites, revealed as strand breaks, are not produced in detectable amounts; and (iii) radiation-induced clustered DNA damage, revealed as additional dsb by treatment with the proteins Nth and Fpg, is significantly dependent upon Γ, in contrast with the independence observed for prompt dsb. From the linearity of the dose−response for the induction of dsb and $n(\text{ess})_{\text{dsb}}$, it is established that these types of damage arise from the interactions with a single radiation track and not an accumulation of multiple lesions arising from more than one radiation track. The formation of dsb by processes arising from multitrack events would show a dependence upon the square of the radiation dose.

The yield of radiation-induced, prompt ssb at $\Gamma = 8$ is similar to that determined[6] in γ-irradiated freeze-dried DNA at ambient temperature and $\Gamma \approx 7$ and that determined[7] in X-irradiated crystalline DNA. The yields of ssb and dsb reported by Baverstock and Will[31] used lyophilized DNA, exposed to a stream of water-saturated gas, are ∼2 times larger than those determined in this study at $\Gamma = 34.5$. Since the yields of prompt ssb and prompt dsb (yields of ssb also shown as G values in Table 1) are only slightly dependent upon Γ, direct energy deposition in the outer hydration layer does not significantly contribute to strand breakage, arising from interactions of radical species produced on ionization of water molecules in this layer (reactions 6c and 7).

$$H_2O \rightsquigarrow e^- + H_2O^{\bullet+} \quad (6a)$$

$$DNA \rightsquigarrow e^- + (DNA^{\bullet+})_D \quad (6b)$$

$$H_2O^{\bullet+} + DNA \rightarrow (DNA^{\bullet+})_{\text{water}} + H_2O \quad (6c)$$

$$H_2O^{\bullet+} + H_2O \rightarrow OH^{\bullet} + H_3O^+ \quad (6d)$$

$$e^- + DNA \rightarrow DNA^{\bullet-} \quad (7)$$

$$(DNA^{\bullet+})_D \rightarrow \text{ssb, dsb, base lesions, clustered damage} \quad (8)$$

$$(DNA^{\bullet+})_{\text{water}} \text{ and } (DNA^{\bullet-}) \rightarrow \text{base lesions, clustered damage} \quad (9)$$

These strand breaks arise from direct ionization of DNA, $(DNA^{\bullet+})_D$ (reactions 6b), probably as a result of ionization of the sugar moiety. Any oxidation of the nucleobases by the sugar radicals must be in competition with their conversion to strand breaks, which are observed. Although the yield of OH•, produced in irradiated hydrated salmon DNA at 77 K, increases when Γ is increased[20] from 9 to 21, it is inferred that any OH•, if produced in the outer hydration layer at 279 K, and $H_2O^{\bullet+}$ (reaction 6a) do not efficiently generate prompt ssb or dsb. It should be remembered that the DNA samples in this study contained Tris (see Materials and Methods) to stabilize closed-circular DNA during sample preparation. The amount of DNA relative to that of Tris is constant at the different levels of hydration so that, to a first approximation, the influence of Tris on DNA damage through radical transfer between DNA and Tris is assumed to be constant. Since Tris is also an OH• scavenger, the majority of any OH• generated in the outer hydration layers could be scavenged by Tris, consistent with the small (∼10%) increase in the yield of ssb when Γ is increased from 8 to 34.5. The electron, which is also formed on ionization of DNA and water molecules, is not thought to be a precursor to strand breakage.[1]

Irradiation of closed-circular DNA in aqueous solution[23,32] at 4 °C in the presence of Tris (see Table 1) followed by incubation at 37 °C or irradiated cellular DNA[33] results in significant yields of additional strand breaks as a result of the presence of heat-labile sites. The presence of heat-labile sites in irradiated plasmid DNA[23,32] is in contrast to the lack of formation of significant amounts of radiation-induced heat-labile

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sites in hydrated DNA in the present study, since the yields of ssb and dsb following heat treatment at 37 °C are the same, within experimental error, as those for prompt strand breakage at all levels of hydration. Since direct ionization of the DNA−water complex does not result in heat-labile strand breakage at $\Gamma < 35$, it is inferred that OH•'s are the probable precursors to the induction of heat-labile sites in DNA[23,32] irradiated in aqueous solution. Tris radicals formed on reaction of OH• and Tris do not induce strand breakage in DNA.[23]

From comparison of the yields of prompt ssb and prompt dsb at $\Gamma = 34.5$ with those induced by γ-irradiated plasmid DNA in aqueous solution containing high concentrations of the OH• scavenger, Tris (see Table 1), the yields of prompt ssb in aqueous solutions are >3 times larger, indicating that a significant fraction of prompt ssb is induced by diffusible water radicals generated in bulk water at these cell mimetic scavenger conditions. In contrast, the yields of prompt dsb (see Table 1) are similar. At a scavenging capacity of 1.4×10^9 s^{-1}, only OH• produced near DNA will interact with DNA, since the mean diffusion distance of the OH• is <4 nm, characteristic of its mean radical diffusion distance estimated[2,34] for the cellular environment around DNA. With dry/hydrated plasmid DNA, the probability of producing a dsb per ssb by γ-irradiation is higher (ssb/dsb ∼10) than that obtained[22,23,32,35−37] in aqueous solutions containing high concentrations of scavenger (ssb/dsb ∼30−40). Some of the slight increase in the yield of prompt ssb at $\Gamma < 8$ may reflect conformational changes of DNA from the A to the B form. The small effects seen in this study are consistent with the similarity of the total radical yield in lyophilised and hydrated calf thymus DNA film,[29,30] although it was concluded that DNA packing rather than conformation is a critical variable in determining the yields of radiation-induced free radicals in DNA.

The yields of base lesions were determined as ess using Nth protein, which excises mainly ring-saturated pyrimidines (e.g. 5,6-dihydrothymine (DHT), thymine glycol, and abasic site (AP site))[38−41] and Fpg protein, which excises[42−44] mainly 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine, 7,8-dihydro-8-oxo-2′deoxyguanine (8-oxoGua), and AP sites. In contrast to the small change of the yield of prompt ssb on the level of hydration, the yields of base lesions, revealed as ssb following a postirradiation treatment with the proteins, increase with increasing hydration of the DNA. The cross reactivity from treatment of irradiated DNA with both enzymes is low. Therefore, the major effect of hydration of DNA is a dramatic increase in the yield of base lesions induced by the direct effects of γ-irradiation. It is proposed that, as the level of hydration of DNA increases, the probability of formation of $H_2O^{\bullet+}$ (reaction 6a) increases. $H_2O^{\bullet+}$ then interacts by one-electron oxidation of the base moieties of DNA (reactions 6c and 9), in competition with its conversion into OH• in reaction 6d or with its deprotonation. That $H_2O^{\bullet+}$ causes one-electron oxidation of base moieties in DNA was previously proposed from EPR studies on irradiated DNA at 77 K.[10] Even at $\Gamma = 34.5$, it is evident that any OH•'s formed do not interact to any significant extent with the sugar moiety to give strand breaks due to their reaction with Tris. It is assumed that the effect of OH• scavenging by Tris on the relative changes of the yields of prompt ssb and *n*(ess) at $\Gamma = 8$ compared with those at $\Gamma = 34.5$ should be similar, assuming that OH•'s are scarcely produced[20] at $\Gamma < 9$. Increasing Γ from 8 to 34.5 results in the yield of prompt ssb increasing by ∼10%, whereas the yield of ess increases by ∼40%. If this small increase in the yield of prompt ssb is due to OH•, then the majority of the additional base lesions seen at $\Gamma = 34.5$ is suggested to be due to interactions of DNA with $H_2O^{\bullet+}$ or electrons produced in reactions 6a and 6b. The contribution of the yield of additional base lesions from electrons, generated in reaction 6a, at $\Gamma > 9$ is expected to increase relative to that from $H_2O^{\bullet+}$ on increasing the value of Γ. The electrons may be trapped by the DNA bases to give base radicals (reaction 7), precursors to base lesions.[10] For instance, at $\Gamma \approx 2.5$, ionization/excitation is limited to DNA and the water molecules associated with the phosphate group or sugar moiety. On increasing Γ from ∼2.5 to 20, energy depositions in the bound water molecules form electrons and $H_2O^{\bullet+}$. Indeed, the total radical yield produced in DNA at 77 K by radiation increases by ∼4 times on increasing Γ from ∼2.5 to 20.[19,20] The major products produced on anaerobic γ-irradiation of DNA at different levels of hydration and at 293 K are DHT and 8-oxoGua,[17] which are substrates for the base excision enzymes, Nth and Fpg, respectively. The yields of these products, based on total sample mass, are independent[17] of the value of Γ. However, if the yields of base damage are related to DNA mass, increases in these yields were observed on increasing Γ, although less than the increase of *n*(ess) on increasing Γ reported here. Artifacts associated with the methods used by Swarts et al.[17] have been reported,[18] especially when estimating the yield of 8-oxoG.

From comparison of the yields of ess at the highest value of Γ, 34.5, with those induced by γ-irradiated plasmid DNA in aqueous solution containing high concentrations of OH• scavengers (see Table 1), *n*(ess) values in aqueous solutions are >5 times larger for both Nth and Fpg lesions, indicating that a significant fraction of ess's are induced by diffusible water radicals generated in bulk water at these cell mimetic scavenger conditions. The ratio of *n*(ess) to the yield of prompt ssb is less than that determined at a high scavenger concentration in aqueous solution by a factor of 1.5. This finding indicates that, although base lesions are more efficiently induced per ssb by diffusible water radicals, the water species produced by direct energy deposition in the hydrated layer are important precursors for the induction of base lesions in DNA, consistent with previous findings.[10,17]

Since the yield of dsb increases following treatment with Nth or Fpg, as shown in Figures 3 and 4, it is proposed that radiation-induced clustered DNA damage $(ess)_{dsb}$'s are produced in irradiated, hydrated DNA. These additional dsb's are not heat-labile sites, as discussed above. As the yields of clustered DNA

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damage per prompt dsb at $\Gamma = 34.5$ are larger (see Table 2) than those determined at high water radical scavenger concentrations in aqueous solution ($\sim 0.8-1$),[23] it is inferred that clustered DNA damage is induced more efficiently by radiation when DNA is hydrated than when DNA is irradiated in aqueous solution under cell mimetic scavenging conditions. These clustered DNA damages arise from generation of a base lesion in the vicinity of either another base lesion or a ssb, on the complementary strand, by energy deposition events from a single radiation track. Conversion of the base lesion(s) into a ssb by the protein probes results in a dsb, if the two lesions are within 10 base pairs of each other, one on each strand.[45] The yield of clustered DNA damage increases with increasing level of hydration. These yields of clustered DNA damage, which include base lesions, are probably lower limits since a neighboring lesion may have an inhibition effect[46−48] on the excision of a base lesion when present within a clustered DNA damage by these enzymes.

Recently, Milligan et al.[49] reported that the majority of additional dsb's induced by γ-irradiation of plasmid DNA in aqueous solution followed by treatment with Fpg or Nth arise from random attack of two $OH^{\bullet}$'s. However, in dry/hydrated DNA, the contribution of $OH^{\bullet}$ to the induction of strand breaks by radiation, as discussed above, is insignificant. Therefore, clustered DNA damage induced by direct effects of radiation arises mainly from direct ionization of the bases or sugar moiety and interaction of the bases with electrons and/or $H_2O^{\bullet+}$.

In summary, prompt strand breaks induced in hydrated DNA arise predominantly from energy deposition in the sugar moiety of DNA, whereas energy deposition in the nucleobases or the hydration layer results in base modifications, but not strand breaks. Energy deposition in the hydration layer results in oxidation of the nucleobases by $H_2O^{\bullet+}$ or reduction of nucleobases by electrons to give base lesions which contribute to the formation of clustered DNA damage. Any $OH^{\bullet}$'s formed in hydrated DNA do not contribute significantly to radiation-induced strand breaks or base lesions at a water content of less than 35 water molecules per nucleotide, possibly due to $OH^{\bullet}$ scavenging by Tris, required to stabilize the plasmid DNA.

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