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Yields of damage to C4′ deoxyribose and to pyrimidines in pUC18 by the direct effect of ionizing radiation

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ABSTRACT

Our mechanistic understanding of damage formation in DNA by the direct effect relies heavily on what is known of free radical intermediates studied by EPR spectroscopy. Bridging this information to stable product formation requires methods with comparable sensitivities, a criterion met by the ^{32}P-post-labeling assay developed by Weinfeld and Soderlind, [Weinfeld,M. and Soderlind,K.-J.M. (1991) ^{32}P-Postlabeling detection of radiation-induced DNA damage: identification and estimation of thymine glycols and phosphoglycolate termini. *Biochemistry*, 30, 1091–1097] which when applied to the indirect effect, detected phosphoglycolate (pg) and thymine glycol (Tg). Here we applied this assay to the direct effect, measuring product yields in pUC18 films with hydration levels (Γ) of 2.5, 16 or 23 waters per nucleotide and X-irradiated at either 4 K or room temperature (RT). The yields of pg [*G*(pg)] for $\Gamma \sim 2.5$ were 2.8 ± 0.2 nmol/J (RT) and 0.2 ± 0.3 nmol/J (4 K), which is evidence that the C4′ radical contributes little to the total deoxyribose damage via the direct effect. The yield of detectable base damage [*G*(B*)] at $\Gamma \sim 2.5$ was found to be 30.2 ± 1.0 nmol/J (RT) and 12.9 ± 0.7 nmol/J (4 K). While the base damage called B*, could be due to either oxidation or reduction, we argue that two reduction products, 5,6-dihydrouracil and 5,6-dihydrothymine, are the most likely candidates.

INTRODUCTION

The effects of ionizing radiation on DNA have been studied for decades as DNA is the critical cellular target responsible for cell killing. Ionizing radiation produces a wide variety of lesions within DNA, such as base modifications, base-free sites, single-strand and double-strand breaks and DNA–protein crosslinks (1,2). These lesions, if unrepaired, may produce detrimental biological consequences, such as mutagenesis, carcinogenesis and even cell death. Consequently, the accurate identification and measurement of these DNA lesions would enable one to better comprehend the chemical mechanistic pathways involved in their formation and in addition, these lesions can also serve as biomarkers of radiation-induced DNA damage.

As a result, assays have been developed to measure the yields of radiation-induced DNA damage with varying degrees of specificity, sensitivity and simplicity. To date, the principal assays used for the detection and quantification of specific DNA lesions have been the analytical techniques of gas chromatography or HPLC coupled with mass spectrometry or electro-chemical detection (3–5), ^{32}P-post-labeling (6) and other assays (7–9). Depending on the lesions being assayed, each technique has its own advantages and disadvantages. The ^{32}P-post-labeling assay, as developed by Weinfeld *et al*. (6), is a highly sensitive technique capable of detecting certain types of radiation-induced DNA damage products at the femtomole level and overcomes many of the problems previously encountered by the other techniques (6,10).

The ^{32}P-post-labeling assay involves the digestion of irradiated DNA by the action of three enzymes, namely, Snake venom phosphodiesterase (SVP), DNase I and shrimp alkaline phosphatase (SAP) as outlined in Figure 1. This approach takes advantage of the fact that certain types of DNA lesions are refractory to these enzymes, preventing the cleavage of the internucleotide phosphodiester linkage immediately 5′ to the site of damage. This incomplete digestion of the irradiated DNA with these enzymes thereby yields dinucleoside monophophates containing these certain types of damage. These dinucleoside monophosphates are readily phosphorylated at their 5′-hydroxyl termini

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by polynucleotide kinase and [γ-^{32}P]ATP. The unmodified bases are recovered as mononucleosides, and are not phosphorylated by the polynucleotide kinase. This technique has been used previously to measure lesions such as phosphoglycolates (pg) and thymine glycols (Tg) (Figure 2), which are products of the indirect effect.

The damage to cellular DNA arises from two sources, the direct effect and the indirect effect. Direct-type damage occurs when the ionizing energy is deposited in DNA itself or transferred into the DNA following ionization of the DNA solvation shell. The DNA solvation shell (Γ) consists of ~20–22 water molecules per nucleotide. Of these, ~15–17 water molecules associate with the DNA nucleoside while ~5 water molecules associate with the phosphate group (11,12). These ~5 water molecules are tightly bound and difficult to remove. The water outside the solvation shell is termed as bulk water. The ionization of the DNA solvation shell produces a water radical cation and an electron. The water radical cation is then involved in two competing reactions: hole transfer to the DNA and the formation of $OH^{\bullet}$ via deprotonation. It has been shown that the formation of $OH^{\bullet}$ is not detected for $\Gamma < 9$–10 but is detected for DNA with $\Gamma > 9$–10 (13,14). The indirect-type damage occurs when the energy is deposited in the water surrounding the DNA (excluding the tightly bound water in the solvation shell) and the subsequent reaction of the radical products ($OH^{\bullet}$, e_{aq} and $H^{\bullet}$), generated in the surrounding water, with DNA. As a point of emphasis, $OH^{\bullet}$ is not formed in DNA samples having $\Gamma < 9$.

Even though direct-type damage contributes to ~50% of the overall DNA damage (15), it is not as well characterized, either quantitatively or mechanistically, as indirect-type damage. Consequently, a better understanding of the DNA lesions induced by the direct effect will lead to greater comprehension of the overall DNA damage chemistry as well as the relative biological effectiveness (RBE). The current study focuses on the direct-type lesions in DNA, comparing these with the indirect-type lesions of pg and Tg. The indirect-type damage product pg is formed when $OH^{\bullet}$ abstracts a hydrogen from C4′ of the DNA sugar moiety in the presence of oxygen (16). The mechanism of Tg formation involves $OH^{\bullet}$ addition to the DNA base moiety in the presence of oxygen (17). Thus, for both these radiogenic lesions, $OH^{\bullet}$ attack is required. Since the direct effect, at extremely low DNA hydration level of $\Gamma \sim 2.5$, cannot involve the formation of $OH^{\bullet}$, the formation of pg and Tg by this pathway is precluded. This assay, therefore, provides a means of comparing sugar and base damage products for the direct versus the indirect effect.

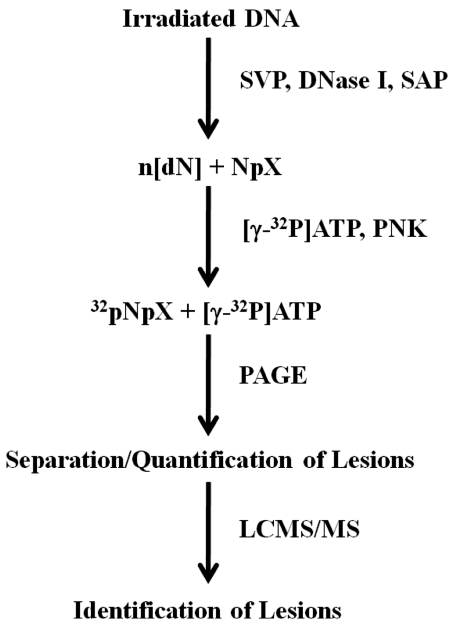


Figure 1. Strategy of post-labeling assay as modified from Weinfeld *et al.* and 'X' in the above represents all damaged nucleosides that are refractory to SVP digestion.

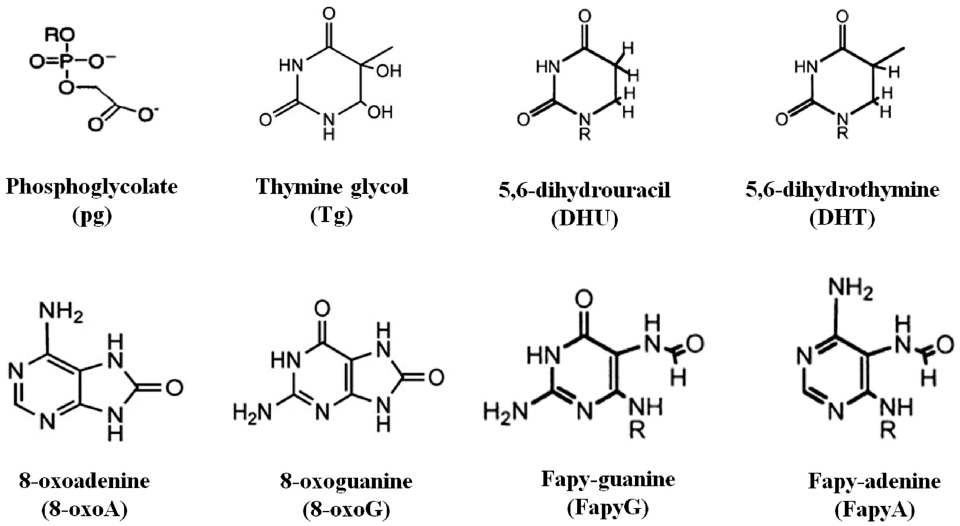


Figure 2. Chemical structures of lesions mentioned in this article.

Three objectives were addressed in our present study: (i) detection of direct-type damage in the bases and deoxyribose groups and comparing these to indirect-type damage, (ii) determination of the influence of DNA hydration on the yields of direct-type damage and (iii) determination of the influence of irradiation temperatures on the yields of direct-type damage. These results provide new insights into the direct-type DNA damage chemistry.

MATERIALS AND METHODS

Enzymes

SVP (Crotalus adamanteus, type I, 0.01 units/μl), DNase I (10 000 units/mg, 2 units/μl) and SAP (1000 DEA units/ml) were purchased from Sigma Chemical Co.; and T4 polynucleotide kinase (10 000 units/ml) was purchased from New England BioLabs Inc. The unit definitions for the enzymes are defined in reference (6).

Sample preparation and X-irradiation

The purification of pUC18 plasmid DNA (2686 bp) was as described previously (18). The concentration of the DNA solution (in 10 mM phosphate buffer, pH ~ 7) was determined by absorbance at 260 nm. Aliquots of the pUC18 DNA solution were then pipetted into open-ended silylated suprasil quartz tubes and dried in sealed chambers either against phosphorous pentoxide or a saturated solution of sodium hydroxide. Under these conditions, it is assumed that DNA contains $\Gamma \sim 2.5$ mol water/mol nucleotide (19). Once dried, the films were weighed and subsequently taken to various higher levels of hydration by allowing them to equilibrate in chambers having a relative humidity of either 84% or 92%. By this protocol, the hydration level of DNA is assumed to be $\Gamma \sim 15$ mol water/mol nucleotide or $\Gamma \sim 23$ mol water/mol nucleotide, respectively (19). The film weights were measured periodically with a Mettler Toledo XP2U or Cahn C-30 Microbalance to an accuracy of ± 0.1 μg and 1 μg, respectively. The accuracy in the terms of percent error of the film weight is less than 0.2% and 2%, respectively. The level of film hydration, Γ (moles of water per mol nucleotide), was calculated from the difference in the weight of the pre- and post-equilibrated films.

At each hydration level, pUC18 DNA films were X-irradiated either at 4 K in a Janis Dewar setup (20) or at room temperature (RT) in a glove box. The X-ray source was a Varian/Eimac OEG-76 H tungsten-target tube operated at 70 keV and 20 mA, and the X-ray beam was filtered by 25 μm thick aluminum foil. Dosimetry was as previously described (21). The dose rate inside the suprasil quartz capillary was 24 kGy/h for 4 K irradiations and 1.1 kGy/min for RT irradiations, taking the attenuation by the quartz into account. The dose regime extended from 0 to a maximum of 6 kGy for the pUC18 DNA films, both at 4 K and RT irradiations. After irradiation at 4 K and RT, the pUC18 DNA films were dissolved in ultra pure distilled water (Gibco, Invitrogen) to 0.5–1 fold weight to volume and then stored at −20°C for later use.

Aliquots of pUC18 DNA solution were used to measure direct-type radiation-induced sugar and base damage products, by employing the post-labeling assay (Figure 1) at the femtomol level (6).

Aqueous samples of unirradiated and ^{60}Co γ-irradiated (0.05 kGy, RT) calf thymus DNA (ctDNA) were included in the post-labeling gels in order to directly compare damage yields between the indirect and direct effect.

Post-labeling assay

For the post-labeling experiments, as previously described (6) and with slight modifications to the protocol therein, 8 ± 0.1 μg of pUC18 DNA film and 8 ± 1 μg of ctDNA were incubated overnight at 37°C with 0.02 units of SVP, 0.2 units of DNase I and 0.2 units of SAP in 30 μl of digestion buffer (10 mM Tris–HC1, pH 7.5, containing 4 mM $MgCI_2$). After overnight incubation, the enzymes were precipitated by addition of three volumes of ice-cold ethanol and followed by centrifugation (10 000*g*) for 15 min. The supernatants were transferred in separate vials and then evaporated while the enzyme precipitates were discarded. The resulting residues, after evaporation, were dissolved in 80 μl of distilled water to obtain approximately a concentration of 0.1 μg/μl. These solutions were heated at 100°C for 10 min to inactivate any residual nuclease and phosphatase activity and then stored at −20°C.

Each phosphorylation reaction mixture (10 μl) contained 1 × T4 polynucleotide kinase buffer (70 mM Tris–HCl, 10 mM $MgCl_2$ and 5 mM dithiothreitol, pH 7.6), 5 units of T4 polynucleotide kinase, 3.3 pmol of [γ-^{32}P]ATP (4500 Ci/mmol, ICN Canada, Montreal, PQ) and 5 μl of enzyme-digested DNA. The samples were incubated at 37°C for 1 h and then the bulk of the excess ATP was consumed by a further incubation at 37°C for 30 min with 1 μl of oligo(dT)$_{16}$ (5 A_{260} units/ml, Integrated DNA Technologies) and 2.5 units of the kinase. After this, an equal volume of formamide loading buffer [90% formamide, 0.02% bromophenol blue and 0.02% xylene cyano1 in 1 × TBE (22)] was added to each sample and half of the material was loaded onto a 20% polyacrylamide/7 M urea gel. The gel electrophoresis equipment, set-up and conditions were the same as previously described (22,23). The radiolabeled products were excised from the gel, after being visualized by autoradiography (Figure 1). The radioactivity per band was counted using a scintillation counter to detect the Cerenkov radiation (without the addition of scintillant).

Chemical yields

For the pUC18 DNA, the radiation-induced damage was calculated at each dose point by normalizing the radioactivity of pUC18 DNA bands against the ctDNA and was based on the presumed target mass consisting of DNA, solvation shell plus counterion, i.e. DNA alone plus 2.5, 16 or 23 waters per deoxynucleotide plus one sodium per deoxynucleotide. From these results, dose–response curves were plotted for each hydration level for sugar and base damage products. The chemical yields were

calculated from the linear region of the dose–response curves of the direct-type radiation induced damage.

RESULTS

Post-labeling assay of direct-type radiation-induced products

The first objective was to determine whether radiation-induced damage in pUC18 DNA, due to the direct effect, could be detected using the post-labeling assay as outlined in Figure 1, when compared to the indirect-type damage in ctDNA. Secondly, to determine whether the direct-type damage migration pattern for pUC18 DNA differed when compared with the indirect-type damage in ctDNA. The post-labeling sequencing gel, as shown in Figure 3, indicates the presence of direct-type damage in pUC18 DNA, sugar and base damage, even at low hydrations, $\Gamma \sim 2.5$ (lanes 2 and 3). In addition, the sugar damage bands (as highlighted in green) for pUC18 DNA (lanes 2, 3, 4 and 5) migrate the same way and have a pattern similar to pg bands of irradiated dilute aqueous solution of ctDNA (lanes 1 and 6), indicating that these pUC18 sugar damage bands are pg products (6). However, the base damage bands (as highlighted in yellow) migrate differently for pUC18 DNA at 4 K and RT irradiations, for hydration levels (Γ) of 2.5 (lanes 2 and 3) and 23 (lanes 4 and 5), when compared to the base damage bands for ctDNA (lanes 1 and 6). This is evidence that direct-type base lesions are produced that differ from the indirect-type lesion Tg.

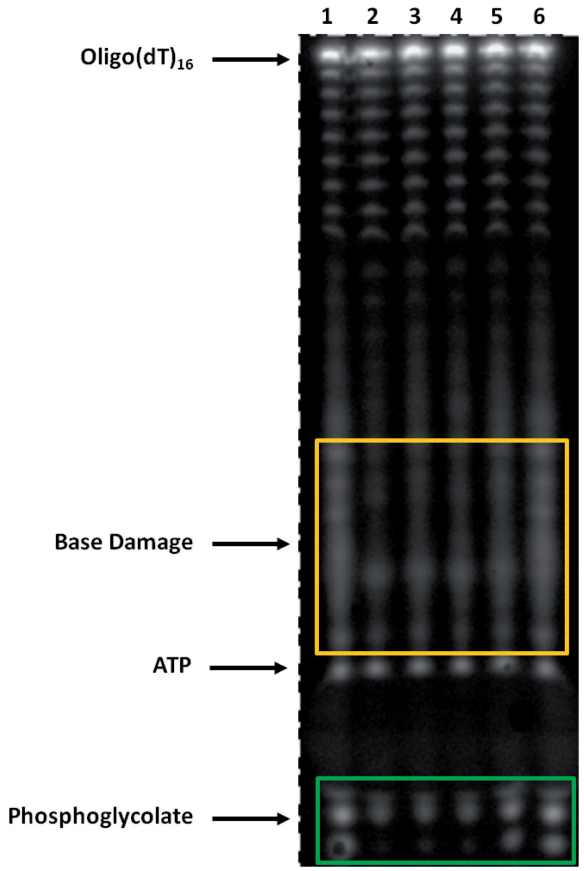


Figure 3. Comparison of X-irradiated pUC18 plasmid DNA with γ-irradiated ctDNA, using post-labeling sequencing gels. Lane 1: ctDNA (0.05 kGy, RT, aqueous solution), lane 2: pUC18 DNA at $\Gamma \sim 2.5$ (3 kGy, 4 K), lane 3: pUC18 DNA at $\Gamma \sim 2.5$ (3 kGy, RT), lane 4: pUC18 DNA at $\Gamma \sim 23$ (3 kGy, 4 K), lane 5: pUC18 DNA at $\Gamma \sim 23$ (3 kGy, RT) and lane 6: ctDNA (0.05 kGy, RT, aqueous solution). The pg and base damage bands highlighted in green and yellow, respectively. The bands on the gel, above the box delineating base damage containing dinucleotides, arise from variable length oligonucleotide impurities in the oligo$(dT)_{16}$ preparation.

Dose–response of direct-type radiation-induced products

In order to quantify the total damage in pUC18 DNA due to the direct effect, and its variation with 4 K and RT radiations and also for various hydration levels, the DNA was subjected to increasing doses of radiation and analyzed by the post-labeling assay. A typical autoradiogram, of the dose–response of pUC18 DNA at $\Gamma \sim 2.5$ and RT irradiation from 0 to 2.8 kGy, is shown in Figure 4. Lane 1, in this figure, is the control for this experiment, which is ^{60}Co γ-irradiated (0.05 kGy, RT) ctDNA in dilute aqueous solution. From the gel in Figure 4, it is evident that the damage products under the pg (as highlighted in green) and base damage regions (as highlighted in yellow) increase with increasing dose. The reproducibility of the gels within the same batch of pUC18 plasmid DNA as well as the batch-to-batch consistency was tested by running multiple replicates of the entire dose range for the DNA samples. Figure 5a and b are examples of the dose–response data for pg and base products, respectively. As seen in Figure 5a, there is no increase in pg, at $\Gamma \sim 23$, with increasing dose for 4 K irradiations while there is an increase for RT irradiations. For base damage products in $\Gamma \sim 2.5$ DNA (Figure 5b), a positive slope to the

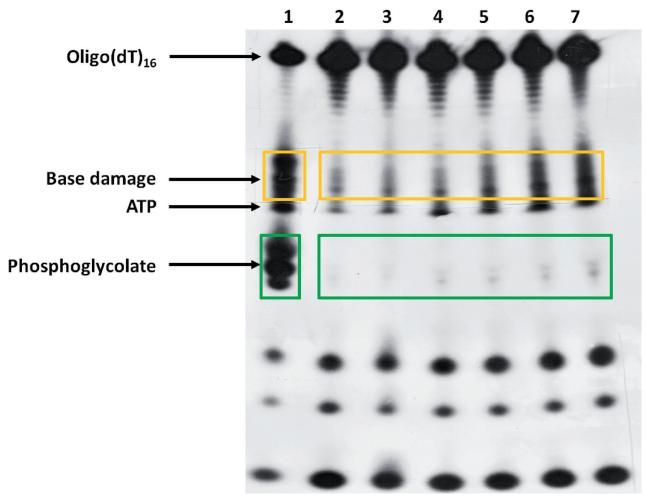


Figure 4. Post-labeling gel showing the dose–response for radiation-induced direct-type damage in pUC18 DNA. Lane 1: ctDNA (0.05 kGy, RT, aqueous solution) and lanes 2–7: pUC18 DNA, $\Gamma \sim 2.5$ (RT), 0.0 kGy, 0.4 kGy, 0.8 kGy, 1.6 kGy, 2.2 kGy and 2.8 kGy, respectively. With increasing dose, the intensity of the radioactive bands in the damage region increases. The pg and base damage bands highlighted in green and yellow, respectively.

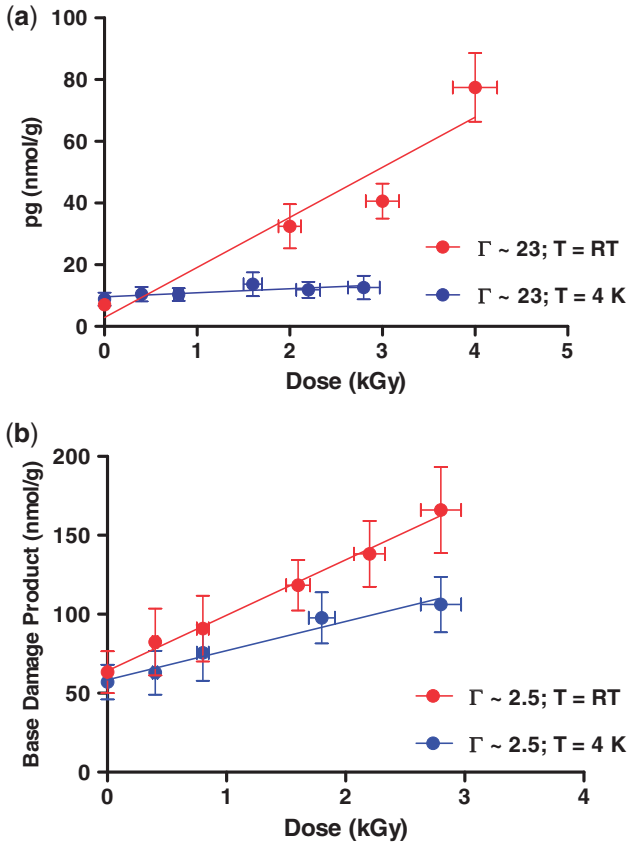


Figure 5. Example of dose–response data used to calculate the chemical yield, *G*. The error bars are ±1 SD based on 2–7 different post-labeling gel experiments per batch of pUC18 DNA. Panel (**a**): pg dose–response for Γ ~ 23, at 4 K (blue) and RT (red). Panel (**b**): Base damage (B*) dose–response for Γ ~ 2.5, at 4 K (blue) and RT (red). The R^2 values and 95% confidence limits (in parentheses) for the fit to a straight line are (i) pg/4 K: $R^2 = 0.64$ (−0.04 to 2.65), (ii) pg/RT: $R^2 = 0.91$ (0.33– 32.1), (iii) B*/4 K: $R^2 = 0.96$ (11.9–25.1) and (iv) B*/RT: $R^2 = 0.99$ (31.2–38.9). The slope of each line is the chemical yield, plotted in Figure 6.

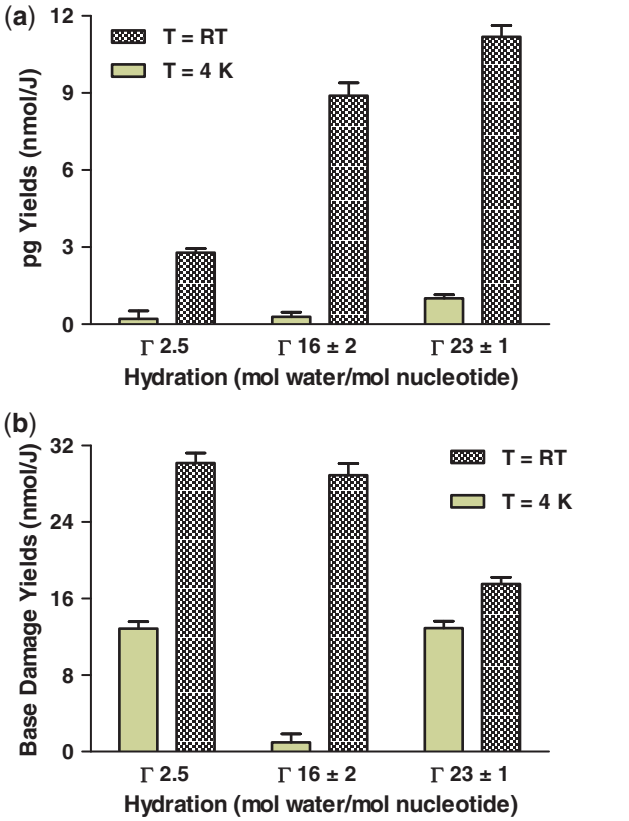


Figure 6. The chemical yields were measured as the slope of the linear region of the dose–response curves and are based on a target mass of DNA, solvation shell plus counterion. Panel (**a**): Chemical yields of pg in samples hydrated to various Γ and irradiated at 4 K and RT. The chemical yields increase significantly as a function of increasing hydration level for RT irradiations. Panel (**b**): Chemical yields of base damage product (B*) in samples hydrated to various hydration levels and irradiated at 4 K and RT. The chemical yields are much larger for RT irradiations than for 4 K. The error bars are ± 1 SD, calculated from 4–5 different post-labeling gel experiments using different batches of pUC18 DNA.

dose–response was observed for 4 K radiations, which increased further for RT irradiations.

The chemical yields for pg and base damage products were calculated from the slope of the linear region of these dose–response curves and were based on a target mass of DNA, solvation shell plus counterion. *G*(pg) for 4 K and RT irradiations and at various Γ were calculated and are represented in the form of a bar graph in Figure 6a. The standard deviations were determined from 4 to 5 different experiments using different batches of pUC18 DNA. *G*(pg), for RT irradiations, increased systematically as the amount of water in the solvation shell was increased; for 4 K irradiations, the yield rises above zero at Γ ~ 23. *G*(pg) was not negligible for Γ ~ 2.5 at RT, contrary to expectations. *G*(base damage) were calculated and are shown graphically in Figure 6b. The *G*(base damage), at Γ ~ 2.5 for 4 K, was significant and increased further for RT irradiations. A detailed explanation for these results is provided in the Discussion.

DISCUSSION

By combining the above findings with the mechanistic constraints imposed by having initiated chemistry via the direct effect, new insights into damage formation in highly concentrated DNA are gained. We first argue that the formation of indirect-type damage products, such as Tg, occurring in these samples is very small at Γ ~ 23, and effectively zero at Γ ~ 2.5. We then describe plausible mechanisms for the dependence of pg and base damage on the level of hydration and temperature of irradiation.

Low probability of indirect-type damage

The yield of $OH^\bullet$ in DNA films is small. At Γ ~ 2.5, where 2–4 waters are tightly bound to the DNA phosphate (11,24), it is known that hole transfer dominates and no $OH^\bullet$ is observed (13,25). In Γ ~ 23 films at 4 K, the yield of $OH^\bullet$ is 23 nmol/J (21,26). From the work of Becker *et al.*, almost all of the $OH^\bullet$ generated in the solvent layer is consumed by the formation of H_2O_2, at 77 K (13).

The little remaining $OH^{\bullet}$ must be divided between reactions with the four bases and the H-atoms bound to the five deoxyribose carbons. Consequently, the yield of any single indirect-type product due to $OH^{\bullet}$ attack is expected to be well below 1 nmol/J. Lastly, while the yield of $OH^{\bullet}$ in $\Gamma \sim 23$ films may be higher at RT than at 4 K, the consumption by H_2O_2 formation coupled with the large number of reactions sites is still expected to result in a very small yield (<1 nmol/J) of any single indirect-type product, such as Tg.

Further evidence against a significant level of $OH^{\bullet}$ comes from studies of intermediate radicals and end-products produced by the direct effect. There is no evidence of $OH^{\bullet}$ adduct radicals in DNA, $\Gamma \sim 2.5$–40, irradiated at 77 K and warmed to > 240K (12,27). In studies of major products formed in films of the oligodeoxynucleotides $d(CTCTCGAGAG)_2$ and $d(GCACGCGTGC)_2$ hydrated to $\Gamma \sim 2.5$ and 23 films and irradiated at RT, there was no measurable dose dependent formation of Tg (28,29).

It should be understood, however, that with regard to RT irradiation of DNA hydrated in the $\Gamma \sim 2.5$–23 range there are, to date, no direct measurements on the yield of $OH^{\bullet}$. The above arguments rely on extrapolation from direct measurements made at low temperature combined with indirect evidence based on DNA product yields at RT.

Formation of pg at 4 K

A pathway proposed for pg formation by the indirect effect involves hydrogen abstraction from C4′ of the DNA sugar moiety by $OH^{\bullet}$, in the presence of oxygen (16), followed by a further eight steps. Out of these 8 steps, 4 of them require radical–radical reactions (2) (Scheme S1 of Supplementary Data). As stated above, there is not a sufficient amount of $OH^{\bullet}$ to give rise to these reactions. In addition, it is known that about 1 radical per 10^5 nucleotides is formed at 6 kGy (30). There are 5372 nucleotides in pUC18 DNA, giving an average of 1 radical per 20 plasmids. These relatively immobile radicals are buried inside a highly compacted supercoiled circular DNA. Any reaction pathway that requires multiple radical–radical reactions, based on the above stated reasons, would be very inefficient in generating pg at 4 K.

One could consider a pathway that is initiated by the deprotonation of the deoxyribose radical cation to form a neutral C4′ radical [see Figure 2.7 in reference (31)], the same radical described above for the indirect effect. From the work of Purkayastha *et al.*, the total free radical yield [*G*(fr)] for $\Gamma \sim 23$ at 4 K is 621 nmol/J and the yield of all trapped radicals on deoxyribose is 11% of the total trapped radicals on DNA (30). From this, the yield of all five deoxyribose radicals is estimated to be 11% of 621 nmol/J, which is ~68 nmol/J at 4 K. Assuming equal probability, the yield of C4′ radical can be estimated to be one-fifth of 68 nmol/J, which is ~14 nmol/J at 4 K. However, warming to RT would result in ~90% of the radicals lost to recombination, thereby leaving the C4′ radical yield to be one-tenth of 14 nmol/J, which is ~1 nmol/J. When the DNA film is solvated, O_2 addition to the C4′ radical would lead to the series of reactions described above for the indirect effect. The expected inefficiencies of radical–radical reactions place the predicted yield of pg via this pathway, therefore, well below 1 nmol/J. We found *G*(pg) to be 0.2 ± 0.3 nmol/J, for $\Gamma \sim 2.5$ at 4 K and 1.0 ± 0.1 nmol/J, for $\Gamma \sim 23$ at 4 K.

Evidence of the importance of hole–hole reactions was obtained by comparing the yield of free base release with the yield of free radicals (32,33). A C4′ carbocation was proposed as an intermediate step (Scheme S2 of Supplementary Data). This pathway consists of (i) formation of deoxyribose radical cation, (ii) deprotonation at C4′ producing the neutral C4′ radical, (iii) oxidation of the C4′ radical by a second hole to produce a C4′ carbocation and (iv) upon dissolution, hydroxide addition leading to ring fragmentation giving pg as one of the products. At low doses, <6 kGy, the second hole must come from within a cluster of ionizations, as proposed previously (32,33). Since there is an increased number of holes due to hole transfer from water to DNA, the yield of holes on the deoxyribose increases as the level of hydration, Γ, increases (34,35). Therefore, the reaction step involving the second hole would be feasible and would explain the observed increase in pg yields as the hydration increases. We note that these radical–radical reactions, which occur through short range mobility within clusters of ionization, are quite different from the radical–radical reactions occurring via diffusion in an aqueous medium as discussed above.

Formation of pg at RT

Similarly, for RT irradiation, we propose that the formation of pg involves a C4′ carbocation intermediate while the contribution from the pathway involving $OH^{\bullet}$ would be negligible. For RT irradiation, the range of hole migration will be substantially greater at RT than at 4 K (36). In addition, as the DNA hydration increases, it stabilizes the DNA duplex, which would in turn increase the range of hole migration. As described for 4 K irradiation, increasing Γ increases the yield of holes on DNA and consequently increases the probability of a second oxidation at C4′. This provides an explanation for the increase in pg yield that occurs when either temperature and/or Γ is increased.

For our studies, we observed *G*(pg) for $\Gamma \sim 2.5$ at RT to be 2.8 ± 0.2 nmol/J and for $\Gamma \sim 23$ at RT to be 11.2 ± 0.4 nmol/J. From the work of Sharma *et al.*, the yield of total free base release *G*(fbr) at RT was reported to be 134 nmol/J at $\Gamma \sim 2.5$ and 212 nmol/J at $\Gamma \sim 23$ (21). Comparing the yields of pg with that of free base release (fbr) and estimating the ratio of *G*(pg)/*G*(fbr), one obtains 2% for $\Gamma \sim 2.5$ and 5% for $\Gamma \sim 23$. With regard to the direct effect, this implies that C4′ oxidation plays a minor role in free base release and/or that pg formation is a minor pathway for the reactions stemming from C4′ oxidation. This observation of pg formation being less significant for the direct effect agrees with the observations of Jones *et al.* (37), who found that irradiation under

conditions that favor direct damage results in a relatively lower yield of pg.

Formation of base product at 4 K

For our studies, we observed G(base damage) for $\Gamma \sim 2.5$ to be 12.9 ± 0.7 nmol/J, at 4 K and 30.2 ± 1.0 nmol/J, at RT. This is indicative of base damage product formed via the direct effect. We call this unidentified product B*. Four major nucleobase products produced by the direct effect are 5,6-dihydrouracil (DHU), 5,6-dihydrothymine (DHT), 8-oxoguanine (8-oxoG) and 8-oxoadenine (8-oxoA), with G(DHU) > G(DHT) (28,29,38,39). Of these, only the dihydropyrimidines (DHPyr) are refractory to SVP (40). We propose that the product B* consists of DHPyr. Since the base products run as dinucleotides in post-labeling gels, seven main products are expected: cytosine-DHU, thymine-DHU, guanine-DHU, adenine-DHU, thymine-DHT, guanine-DHT and adenine-DHT. Formation of cytosine-DHT is unlikely because electron trapping by Cyt dominates over trapping by an adjacent Thy (29). The proposed mechanism for DHPyr formation is one-electron reduction, followed by proton addition, followed by a second electron addition forming a carboanion that may protonate in the solid state or upon dissolution [Scheme 1, also see reference (29)]. This two-electron-reduction mechanism is, in two respects, the same as that proposed for pg formation. (i) It requires combination of two like charges. Two holes must combine to yield pg and two electrons must combine to yield DHPyr. (ii) Proton transfer from (or to) the initial oxidized (or reduced) radical makes the second redox event energetically favorable.

Combination reactions annihilate > ~600 nmol/J of the initial radicals formed in DNA, at 4 K (41). The larger fraction of these entails recombination of a hole and electron, leaving no damage. But the smaller fraction, due to electron–electron (e–e) or hole–hole combination reactions, forms irreversible damage before the DNA is dissolved. What few radicals remain in solid state DNA at RT [<~60 nmol/J (42)] will not give rise to DHPyr upon dissolution because dominating reactions due to O_2 addition lead to other products.

A two-electron-reduction mechanism (Scheme 1) can explain our finding that base product yield decreased from 12.9 ± 0.7 nmol/J to 1.0 ± 0.9 nmol/J, when Γ was increased from 2.5 to 16. Earlier work has shown that the total free radical yield [G(fr)] dependency on DNA hydration follows an S-shaped curve, increasing by 2-fold between $\Gamma \sim 7$ and 15 (34). The yield of trapped radicals decreases as a consequence of an increase in yield of combination events; thereby, the concentration of excess electrons (EE) on DNA decreases 2-fold. Given that reaction of two EE follows the square of the EE concentration, a 4-fold decrease in e–e combination reactions (Reaction 4) is predicted. This is in reasonable agreement with the observed decrease of 12 nmol/J, when increasing Γ from 2.5 to 16.

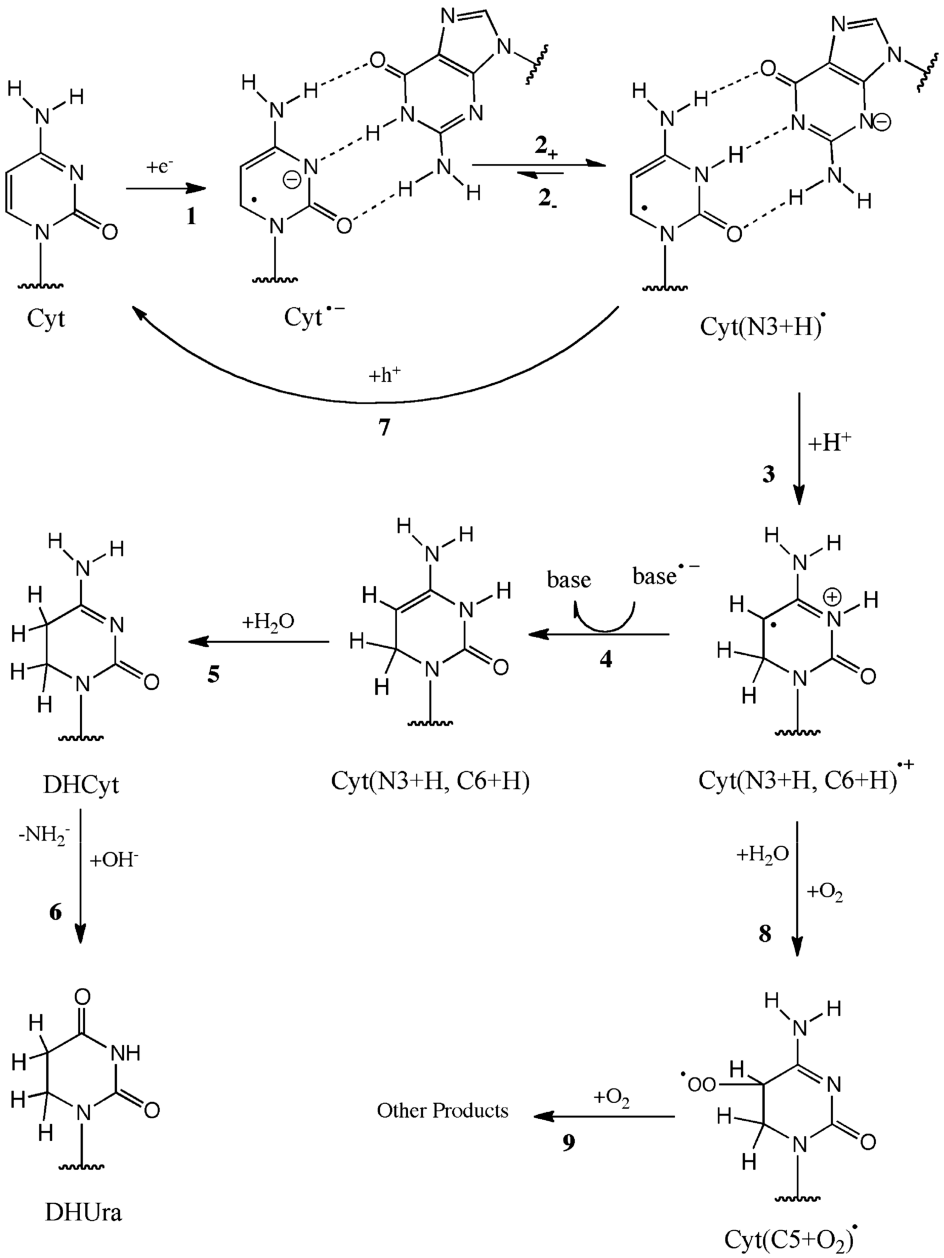
Following the above-described decrease in base product yield, there was an increase from 1.0 ± 0.9 nmol/J to 12.9 ± 0.7 nmol/J, when Γ was increased from 16 to 23. This too can be explained by Scheme 1. Water in the outer solvation shell is a source of electrons that end up attaching to DNA. This does not hold true for holes ($H_2O^{\bullet+}$) in the outer shell, which instead of transferring to DNA, form $OH^{\bullet}$. As a consequence, a surplus of EE relative to holes (Reaction 1 versus 7) accumulates on DNA and that surplus increases in going from Γ of 16 to 23. At $\Gamma \sim 23$, the gap between holes and EE transferred to DNA is ~46 nmol/J. If all of the additional EE were consumed at two electrons per DHPyr, this would account for a maximum increase in DHPyr yield of ~23 nmol/J. This is in reasonable agreement with the 12 nmol/J increase that was observed.

Formation of base product at RT

The range of an EE in DNA at 4 K is limited primarily by the fast rate of proton transfer from the N1 of Gua to N3 of the Cyt radical anion (Reaction 2_+). Because of this, EE trapping is very efficient. At RT, it has been shown that while Cyt decreases the efficiency of EE transfer through a stretch of Thy, transfer does occur (43). This difference between 4 K and RT is very likely due to there being sufficient thermal energy at RT to drive reverse proton transfer (Reaction 2_-), from the protonated Cyt radical anion back to Gua. Thus, the range of EE in DNA is greater at RT. In addition, the rate of irreversible carbon protonation for both one-electron reduced Thy and Cyt will be faster at RT (44). The increased EE range and increased rate of irreversible carbon protonation work together to increase the yield of DHPyr.

Here, we found G(B*), for $\Gamma \sim 2.5$ at RT, to be ~30 nmol/J. For two duplex oligodeoxynucleotides (ODN), namely, $(GCACGCGTGC)_2$ and $(CTCTCGAGAG)_2$ irradiated at RT, the yield of DHPyr is as follows. For CTCTCGAGAG, G(DHT + DHU) is 181 nmol/J at $\Gamma \sim 2.5$ and 54 nmol/J at $\Gamma \sim 15$ (29). For GCACGCGTGC, G(DHT + DHU) is 57 nmol/J at $\Gamma \sim 2.5$ (29). This downward trend, as Γ increases, is the same as that observed for product B* from our pUC18 DNA results. Comparing the yields of product B* with that of DHPyr in ODNs, the ratio of G(B*)/G(DHPyr) is $\sim 30/181 \approx 1/6$ for CTCTCGAGAG at $\Gamma \sim 2.5$ and $\sim 30/57 \approx 1/2$ for GCACGCGTGC at $\Gamma \sim 2.5$. The less interesting possibility is that SVP only fails to cleave the phosphodiester bond 5′ to 16–50% of the DHPyr lesions (40). While the more interesting possibility is that, in highly polymerized and highly compacted DNA, i.e. plasmid versus ODNs, the efficiency of the two one-electron reduction pathway is reduced by 3–6 fold. The radical yields for ODNs and pUC18 DNA are comparable at 4 K (28,30) but have not been measured at RT. If the yield of radicals produced by protonation of the C5–C6 bond of the one-electron reduced pyrimidines is relatively high for pUC18 irradiated at RT, this would explain the smaller yield of product B*.

Lastly, we note the findings of Falcone *et al.* (38) based on the use of GCMS to determine the yields of DHPyr and H_2 in salmon sperm DNA, freeze dried, variably hydrated and γ-irradiated at RT. The large yields (41–108 nmol/J) for DHPyr and low yield (14–18 nmol/J)



Scheme 1. DHUra formation via two one-electron reductions. Reaction 1: one-electron reduction of Cyt gives the cytosine radical anion ($Cyt^{\bullet -}$), which rapidly protonates via proton transfer from N1 of Gua (2_+), even at 4 K, to give $Cyt(N3+H)^{\bullet}$; as T approaches RT, the reverse reaction (2_-) is activated. Reaction 3 occurs at $T > \sim 200$ K where a second protonation gives the radical cation $Cyt(N3+H, C6+H)^{\bullet +}$. Reaction 4: electron transfer to this radical from a nearby base radical anion yields doubly reduced Cyt. Reaction 5 occurs when the sample is dissolved in water, conversion results in DHCyt, which deaminates to form DHUra, Reaction 6. Competing with 3 is recombination of the EE with a hole to give Cyt, Reaction 7. Competing with 4, but only after dissolution, is peroxyl radical formation by oxygen addition, Reactions 8–9. (29).

for H_2 led them to conclude that in forming DHPyr, DNA consumes EE and thereby competes with H_2 formation. In other words, DNA scavenges a large fraction of the intra-spur electron reactions. Their conclusions are consistent with our hypothesis that B* is DHPyr.

CONCLUSION

The chemical yields of phosphoglycolate, a product due to oxidation at C4′ of the deoxyribose, at $\Gamma \sim 2.5$ was found to be 2.8 ± 0.2 nmol/J for RT irradiations and 0.2 ± 0.3 nmol/J for 4 K irradiations. From this, we conclude that oxidation at C4′ contributes only a small fraction of the total deoxyribose damage via the direct effect. The yield of detectable base damage [G(B*)] at $\Gamma \sim 2.5$ was found to be 30.2 ± 1.0 nmol/J (RT) and 12.9 ± 0.7 nmol/J for 4 K irradiations. Given the paucity of water in these samples, we suggest that a major fraction of product B* consists of 5,6-dihydrothymine and 5,6-dihydrouracil stemming from a reaction pathway consisting of one-electron reduction, followed by protonation and a second one-electron reduction.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Schemes 1 and 2.

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