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January 17, 1994

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Angela Hart
Contract Specialist
General Acquisitions Branch
Procurement & Contracts Division
Department of Energy, Field Office, Oakridge
P.O. Box 2001
Oakridge, TN 37831

RE: Final report grant #DE-FG05-86ER60464.

Dear Ms. Hart:

Enclosed is the final progress report on my DNA grant entitled, "Direct Assay of Radiation-Induced DNA Base Lesions in Mammalian Cells". Also enclosed are five reprints of the two papers published since the last progress report. Marty Dozier in the Controller's Office will forward a Final Budget Expenditure Summary by January 31, 1994. I have filled out the form 1338.15 that you faxed to Larry Smith in December and attached it to this report. I anticipate 3-4 additional papers will be published on this work. I shall forward five reprints of each when they are received.

I believe this completes all of my reporting requirements for this grant. If any additional information is needed, please contact me at 910-716-6189 by phone or 910-716-2029 by FAX, and I will supply it immediately.

Thank you for your cooperation in this matter.

Sincerely yours,

Kenneth T. Wheeler

Kenneth T. Wheeler, Ph.D.
Professor and Director
Experimental Radiation Oncology
Department of Radiology

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PART I *(DOE, DOE Contractors, Grantees, and Awardees complete)*

A. Product/Report Data

1. (Award) Contract No. DE-FG0586ER60464

2. Title Direct Assay of Radiation-Induced DNA Base Lesions in Mammalian Cells

3. Product/Report Description

☒ a. Report *(Complete all that apply)*
(1) ☒ Print ☐ Nonprint *(specify)*
(2) ☐ Quarterly ☐ Semiannual ☐ Annual ☒ Final
☐ Topical ☐ Phase I ☐ Phase II
☐ Other *(specify)*
Dates covered 11/1/91 thru 10/31/93

☐ b. Conference/Meeting/Presentation *(Complete all that apply)*
(1) ☐ Print ☐ Nonprint *(specify)*
☐ Published proceedings
☐ Other *(specify)*
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Location *(city/state/country)*
Date(s) *(m/d/y)* / / thru *(m/d/y)* / /
Sponsor

☐ c. Software—Additional forms are required. Follow instructions on the back of this form.

☐ d. Other *(Provide complete description)*

B. Patent Information

Yes	No	
☐	☒	Is any new equipment, process, or material disclosed? If yes, identify page numbers
☐	☒	Has an invention disclosure been submitted? If yes, identify the disclosure number and to whom it was submitted. Disclosure number Submitted to
☐	☒	Are there patent-related objections to the release of this STI product? If so, state the objections.

C. Contact *(Person knowledgeable of content)*
Name Kenneth T. Wheeler, Jr.
Phone (910) 716-6189; FAX (910) 716-2029
Position Professor & Director
Organization Bowman Gray School of Medicine Winston-Salem, NC 27157

PART II *(DOE/DOE Contractors complete/or as instructed by DOE contracting officer)*

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1021396

FINAL PROGRESS REPORT
DOE Grant DE-FG05-86ER60464
September 1, 1991 - November 1, 1993

BACKGROUND:

This progress report covers the period from September 1, 1991 - November 1, 1993. This period encompasses the last year of the grant for which money was awarded and an extension of the grant for one year without funds in order to complete as many of the goals of this project as was possible.

SPECIFIC AIMS:

Because of large budget cuts in the last two years of the grant, the specific aims were reduced to the following two:

1. To develop procedures for quantitatively isolating radiation-induced DNA base damage products from mammalian cells.

2. To identify and quantitate the types and yields of base lesions produced in irradiated salmon sperm DNA under dry, hydrated, and solution conditions.

PROCEDURES FOR ISOLATING AND QUANTITATING BASE DAMAGE PRODUCTS:

DNA Isolation Procedure:

Reproducible isolation of DNA from modest numbers of mammalian cells or from small pieces of tumor and normal tissue has been a significant problem. In the past year, the reproducibility has been substantially improved with the use of the A.S.A.P. genomic DNA isolation kit from Boehringer Mannheim Corporation. Samples containing 1 - 5 x 10^7 cells now yield 50 - 100 μg of DNA that is suitable for the isolation of damaged bases. In fact, the buffy coat from 10 ml of blood provides sufficient DNA to measure background levels of base damage from untreated nucleated white cells. Minor, but important, modifications have been made to the recovery procedures that increased the yield of DNA substantially. It is now feasible to perform radiation studies on < 10^8 cells. Therefore, DNA base damage studies can be performed in both tissue culture cells and tissues.

Formic Acid Hydrolyses Procedure:

Gas chromatography/mass spectrometry (GC/MS) is a promising method for performing both qualitative and quantitative analysis of DNA base damage products. This method commonly uses a hot (150°C) formic acid hydrolysis step to degrade the DNA to individual free bases. It has been suggested that several of the DNA base products may be chemically altered by the formic acid hydrolysis; thereby, affecting the quantitation of these products. Because the overall goal of this research program requires quantitation of radiation-induced DNA base damage in mammalian cells, several formic acid hydrolysis procedures were examined to ascertain the impact of these procedures on the quantitation of a number of DNA base damage products. Where effects were noted, alternative procedures were explored in an attempt to accurately quantitate the various base damage products.

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Overproduction of human Mn-superoxide dismutase modulates paraquat-mediated toxicity in mammalian cells*

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Received 5 September 1991; revised version received 7 October 1991

Manganese superoxide dismutase (MnSOD) is a nuclear encoded mitochondrial matrix enzyme that functions to scavenge superoxide radicals. The human MnSOD cDNA under the transcriptional control of a human β-actin promoter was introduced into mouse C3H10T$_{\frac{1}{2}}$ cells by cotransfection with a recombinant plasmid containing the Neo^R selectable marker. C3H10T$_{\frac{1}{2}}$ transformants (C3H-SOD) were obtained that expressed high levels of authentic enzymatically active human MnSOD. Overexpression of the MnSOD gene did not affect the protein levels of CuZnSOD, catalase (CAT), or glutathione peroxidase (GPX) in the transformants. Treatment of cells with paraquat was less toxic to the C3H-SOD cells than to the control cells. These results are consistent with the possibility that superoxide radicals are mediators of paraquat cytotoxicity.

Superoxide dismutase; Gene dosage; Mitochondrion; Oxidative stress; Paraquat

1. INTRODUCTION

The herbicide paraquat (methyl viologen; 1,1′dimethyl-4,4′-bipyridinium dichloride) has been responsible for a large number of deaths after accidental or intentional ingestion or injection [1]. The toxic effect of paraquat is believed to be mediated by cytotoxic oxygen free radicals that are produced intracellularly during cyclic oxidation and reduction of paraquat by metabolic processes within the cells [2,3].

It has been shown that treatment of cells or animals with increasing concentrations of paraquat leads to increased cellular content of both Mn- and CuZn-containing superoxide dismutases (MnSOD and CuZnSOD), catalase (CAT), and glutathione peroxidase (GPX) [4,5]. MnSOD and CuZnSOD are protective enzymes responsible for maintaining low levels of superoxide radicals within cells. MnSOD is localized mainly in the mitochondrial matrix [6], whereas CuZnSOD is localized in the cytosol [7]. Dismutation of superoxide radicals yields hydrogen peroxide, which is subsequently detoxified by CAT or GPX.

It is generally thought that the toxicity of superoxide radicals stems from their ability to interact with hydrogen peroxide to generate highly reactive singlet oxygen ($^1O_2$) and/or hydroxyl radicals (OH) [7–10]. Thus, it is possible that the observed increases in antioxidant enzyme systems following exposure to agents that generate superoxide radicals are necessary for the protection of mammalian cells.

To examine the role of MnSOD in the defense against paraquat toxicity, human MnSOD cDNA was introduced into mouse C3H10T$_{\frac{1}{2}}$ cells with a recombinant plasmid containing the Neo^R selectable marker. The selected G418 transformants produced authentic, enzymatically active human MnSOD. The effects of MnSOD overproduction on paraquat-induced cytotoxicity in these cells was then examined.

2. MATERIALS AND METHODS

2.1. *Construction of recombinant plasmid expressing human MnSOD*

To construct the human MnSOD expression vector, the human MnSOD cDNA [11] fragment flanked by the *Eco*RI restriction site was initially blunt-ended with mung bean nuclease, ligated to *Sal*I linkers followed by digestion with *Sal*I, and then inserted into the *Sal*I site of the human β-actin expression vector pHβAPr- [12] (generously provided by Dr. Larry Kedes of the University of Southern California, Los Angeles, CA). All recombinant DNA procedures were performed according to the methods described by Maniatis et al. [13].

2.2. *Transfection and selection of cell lines*

The mouse embryonic fibroblast C3H10T$_{\frac{1}{2}}$ clone 8 was maintained in Eagle's basal medium (BME) supplemented with 10% fetal calf serum (FCS) and 10 μg/ml gentamicin. Subconfluent cultures in 100-mm dishes were cotransfected with the MnSOD expression plasmid (10–20 μg) and the pSV2-Neo^R plasmid [14] at a molar ratio of 10:1 by using Lipofectin (BRL). After 48 h, G418 (Gibco) at 400 μg/ml was added to the cells for selection. G418-resistant clones were maintained in complete medium. Control cells were transfected with pSV2-Neo^R

*Preliminary results of this study were presented at the Eighty-second annual meeting of The American Association for Cancer Research, 1991.

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alone and maintained under identical conditions. For detection of MnSOD, cells were grown to confluence in medium without antibiotics.

2.3. Detection of MnSOD RNA

Levels of RNA were detected by Northern analysis. Total RNA was isolated by the guanidine isothiocyanate method [15], enriched for poly(A)$^+$RNA [16], size-separated by formaldehyde-agarose (1.1%) gel electrophoresis, and transferred to a nitrocellulose filter. The filter was baked at 80°C and hybridized to a ^{32}P-labeled MnSOD cDNA [17] in 50% formamide at 42°C. The filter was washed twice, for 30 min in 2 × SSC, 0.1% SDS at room temperature, and twice in 0.1 × SSC, 0.1% SDS at 65°C for 1 h.

2.4. Detection of immunoreactive MnSOD, CAT and GPX protein

The amount of immunoreactive MnSOD, CAT and GPX protein was measured by a Western blotting procedure as previously described [18]. Briefly, cell homogenates were electrophoresed on a 12.5% polyacrylamide slab gel following pre-treatment with SDS and β-mercaptoethanol at 100°C. Protein from the gel was transferred to a nitrocellulose filter and blocked for 1 h at room temperature in Tris-buffered saline containing 0.05% polysorbate 20 (Tween-20) and 20% FCS. The nitrocellulose filters were then incubated with a 1:5000 dilution of rabbit anti-human MnSOD antibody followed by biotinylated secondary IgG and immunoperoxidase staining.

2.5. Detection of SOD activity

The activity of SOD was detected by the nitroblue tetrazolium staining method [19]. Cells were homogenized in 10 mM Tris (pH 7.0)/10 mM dithiothreitol/0.1% Triton X-100 and centrifuged at 10 000 × g for 2 min. Samples were electrophoresed in polyacrylamide gels, consisting of a 5% stacking gel (pH 6.8) and a 10% running gel (pH 8.8).

To visualize SOD activity, gels were first incubated in 2.5 mM nitroblue tetrazolium in H_2O for 15 min followed by 0.028 mM riboflavin/30 mM TEMED/50 mM potassium phosphate (pH 7.8) for 15 min in the dark, washed in deionized water, then illuminated under fluorescent light until clear zones of SOD activity were distinctly evident.

2.6. Immunochemistry of cultured cells

Flasks with confluent monolayers were prepared for immunostaining with polyclonal antibodies against MnSOD, CuZnSOD, CAT, and GPX supplied by Dr. Larry W. Oberley as described earlier [20,21]. Staining was performed by the avidin–biotin–peroxidase complex using a universal staining kit (Biogenex Co., Dublin, CA). To preserve the morphology of cells as they were in vitro, cells were not detached from the flasks, but were fixed and stained in situ. The cells were fixed with 10% buffered formalin (3.7% formaldehyde) solution at room temperature for 10 min, rinsed with phosphate-buffered saline (PBS), and postfixed with absolute methanol (−10°C) for 10 min.

Immunostaining was performed following a routine protocol. Cells were rinsed with PBS, and endogenous peroxidase was blocked by incubating for 5 min with a 5% solution of hydrogen peroxide in methanol. The monolayer in each 25-cm^2 flask was divided into 4 areas, 1 for each of the antibodies to antioxidant enzymes and 1 for nonimmune (negative) control serum. Nonspecific antigenic sites were blocked with normal goat serum for 30 min. Excess serum was then removed, and the primary antibodies were incubated overnight at 4°C. The flasks were rinsed and secondary antibody (biotinylated goat anti-rabbit) was applied for 30 min. Label (avidin–peroxidase complex) was added for 30 min. Color was developed by incubating with diaminobenzidine (DAB) (0.25% in PBS), together with a 3% aqueous solution of H_2O_2 for 3–6 min.

2.7. Assay of paraquat cytotoxicity

G418-resistant cells and parental cells were grown in BME supplemented with 10% FCS. 24 h before the application of paraquat, cells were seeded in 100-mm dishes (300 cells/dish). Paraquat at various concentrations was applied in quadruplicate, and cells were further incubated. Paraquat was removed after 48 h, cells were rinsed with PBS, and medium without paraquat was added. Cells were allowed to grow for an additional 10–12 days. Cell survival was measured by the ability of C3H10T$^+$ cells to form colonies in vitro.

3. RESULTS

3.1. Generation of C3H10T$^+$ cells that express elevated levels of human MnSOD

The mouse embryonic fibroblast C3H10T$^+$ cells were transfected with plasmid pSV-Neo^R, a vector containing a neomycin-resistant gene, or transfected with plasmid pSV-Neo^R plus the human MnSOD expression vector, and then selected for resistance to the antibiotic G418. Five colonies of resistant cells from each transfection were pooled. Integration of the transfected plasmid DNA into cellular DNA was examined by Southern blot analysis [22] (data not shown). RNA blot analysis demonstrated the expression of the human MnSOD sequence in C3H10T$^+$ cells that were transfected with the human MnSOD vector (C3H-SOD), but not in cells transfected with pSV-Neo^R (C3H-NEO) (Fig. 1).

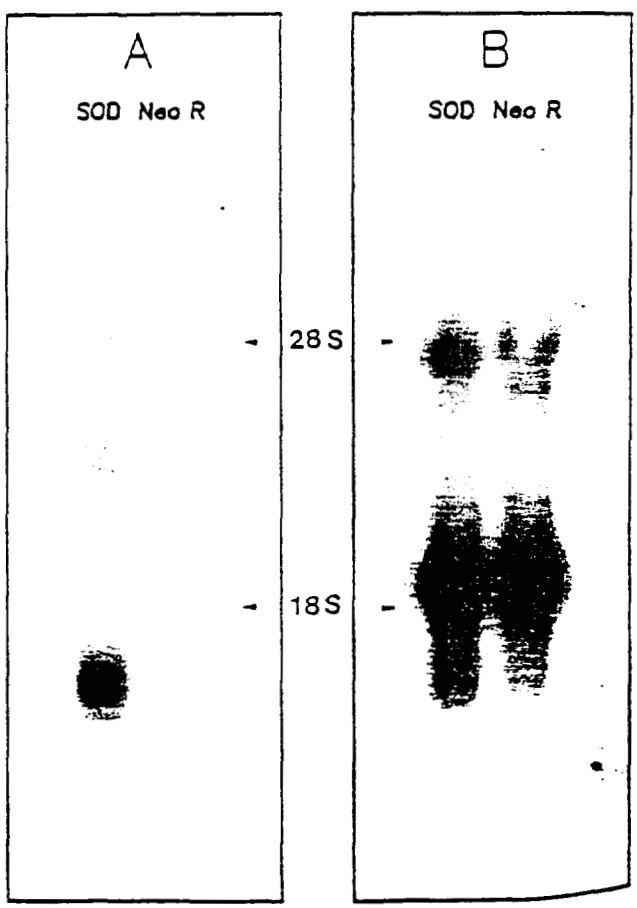

Fig. 1. Northern analysis of cellular RNA. (A) Poly(A$^+$)RNA from C3H-NEO and C3H-SOD cells was separated on a 1.1% formaldehyde agarose gel and, following transfer to nitrocellulose, probed with a ^{32}P-labeled MnSOD cDNA. (B) The same blot was probed with a β-actin.

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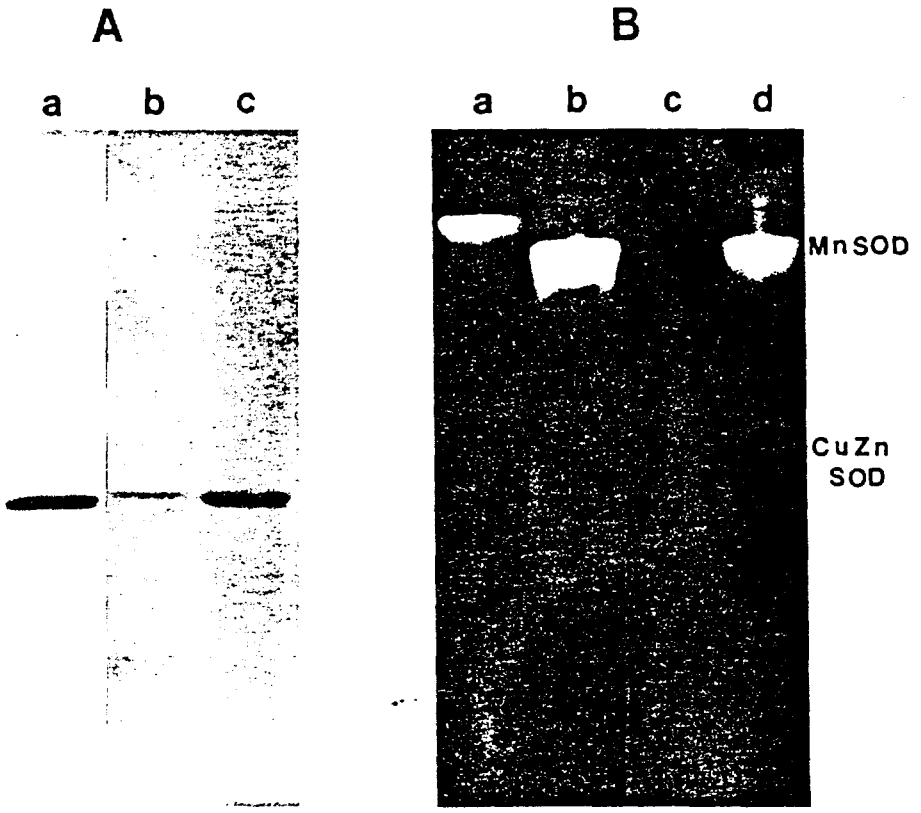

Fig. 2. (A) Western analysis of human MnSOD. Samples were prepared and electrophoresed through polyacrylamide gels, electrotransferred, and then immunologically stained. (Lane a) 1 μg of purified chicken liver MnSOD; (lane b) 250 μg of cell homogenate from C3H-NEO; (lane c) 250 μg of cell homogenate from C3H-SOD cells. (B) Native polyacrylamide gel stained for SOD activity. Samples were electrophoresed through a nondissociating riboflavin gel and stained for SOD activity by the photoinduced NBT reaction. (Lane a) 1 μg purified chicken liver MnSOD; (lane b) 1 μg purified human kidney MnSOD; (lane c) 200 μg of cell homogenate from C3H-NEO cells; (lane d) 200 μg cell homogenate from C3H-SOD cells.

Protein blot analysis using anti-human MnSOD antiserum showed increased MnSOD protein in C3H-SOD cells compared to that in C3H-NEO cells (Fig. 2A). The endogenous levels of CuZnSOD, CAT and GPX were unchanged in C3H-SOD and C3H-NEO cells as examined by Western blot staining (data not shown).

To verify further that the overexpressed MnSOD protein in C3H-SOD cells is active, the SOD activity gel assay was performed. Equal amounts of protein from C3H-NEO cells and C3H-SOD cells were separated on a 10% native polyacrylamide gel, and SOD activity was detected by the nitroblue tetrazolium staining method (Fig. 2B). A species of enzymatically active MnSOD which co-migrated with the purified human MnSOD on the activity gel was found only in C3H-SOD cells.

Immunohistochemical stained cells showed intense granular cytoplasmic staining for MnSOD in C3H-SOD cells, but only slight staining in C3H-NEO and C3H10T½ cells (Fig. 3).

3.2. *Cellular effects resulting from overproduction of human MnSOD*

If the toxic action of paraquat is mediated through the generation of superoxide radicals [2,3], then the cytotoxic effects of paraquat on C3H-SOD, C3H-NEO, and C3H10T½ cells should be different. When the cells were treated with paraquat for 48 h and the extent of survival was determined, the C3H-SOD cells were much more resistant to killing by paraquat than either the C3H-NEO or C3H10T½ cells. The C3H-NEO or C3H10T½ cells were killed by paraquat to the same extent (Fig. 4).

4. DISCUSSION

The toxic mechanism of paraquat in cells has been linked to the production of excess superoxide radicals [1–4]. If this proposed mechanism for the toxic action of paraquat is valid, then increased intracellular superoxide dismutase should protect cells against paraquat toxicity.

The data presented here demonstrate that an increase in MnSOD activity can protect cells against paraquat toxicity. Thus the role of superoxide radicals in the toxic action of paraquat and a role of MnSOD in the defense against paraquat cytotoxicity are strongly supported by our data. Earlier studies that also support this conclusion include the finding that induction of SOD activity renders bacteria, plants and animals more resistant to paraquat toxicity [4,23,24]. However, in these studies

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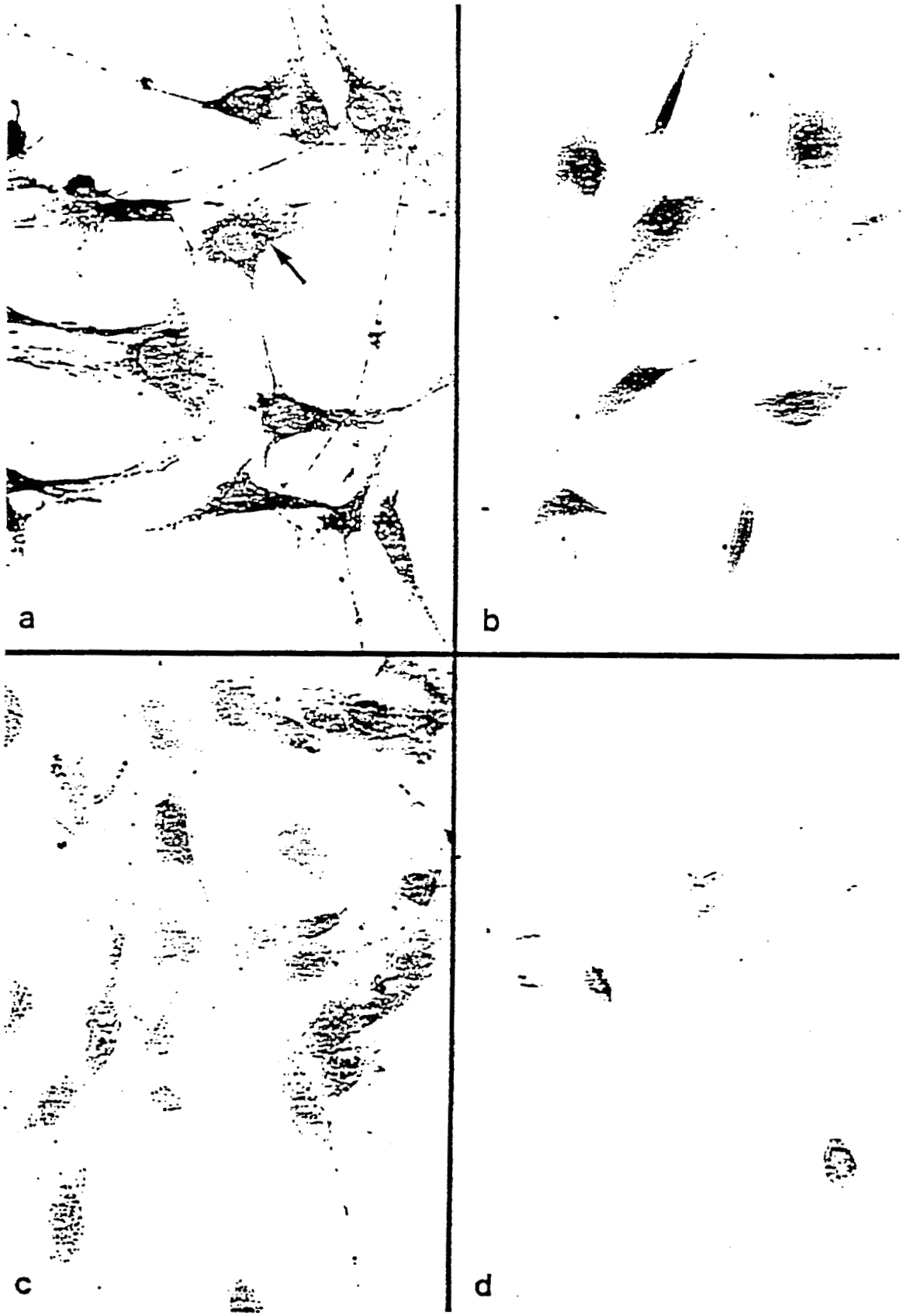

Fig. 3. Immunoperoxidase staining for MnSOD. (a) C3H-SOD with anti-MnSOD antibodies; (b) C3H-NEO with anti-MnSOD antibodies; (c) C3H-10T with anti-MnSOD antibodies, (d) C3H-SOD with nonimmune serum. Magnification ×750. Arrow indicates granular cytoplasmic staining for MnSOD.

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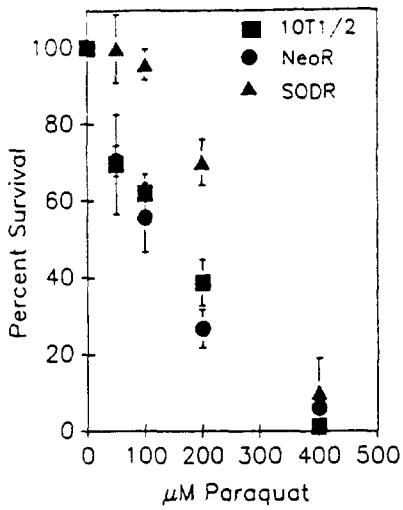

Fig. 4. Dose-response of cell killing by paraquat. Cells were incubated with indicated concentration of paraquat for 48 h. Survival was determined by a colony formation assay.

either the changes in other antioxidant enzymes were not determined, or the induction of SOD was accompanied by an induction of CAT or GPX. Our immunological analysis suggested that overexpression of human MnSOD in the C3H-SOD cells has no effect on the expression of CuZnSOD, CAT, or GPX. In addition, mammalian cells overexpressing human CuZnSOD also show resistance to paraquat [4,24,25]. However, in one study, overexpression of CuZnSOD was accompanied by a decrease in endogenous MnSOD acitivity, and a direct correlation between CuZnSOD activity and resistance to paraquat could not be demonstrated [26]. Thus, our result is the first to demonstrate clearly that MnSOD can be responsible for the protection of mammalian cells from paraquat. Consistent with our results is the study by Wong et al. [27] in which transfection of the MnSOD gene did not cause any change in CuZnSOD, CAT, or GPX levels in the transfected cells and led to cellular resistance to killing by tumor necrosis factor (TNF). Since several lines of evidence implicate oxygen radicals as mediators of TNF-induced cell injury [28–30], the significance of MnSOD in the defense against oxygen radical toxicity in mammalian cells is further confirmed by these latter studies.

The finding that increased MnSOD levels without concurrent increases in CuZnSOD, CAT, or GPX protect cells from the cytotoxicity of paraquat should not be taken as evidence that CAT and GPX are not involved in protecting cells against paraquat toxicity. It has been found that certain clones of cells that exhibit higher CuZnSOD activity are even more sensitive to paraquat [25]. More recent results demonstrate that the levels of endogenous glutathione peroxidase as well as CuZnSOD may also contribute to the tolerance of paraquat by cells [26]. Therefore, the balance of the intracellular redox state may be the key factor in the well-being of cells under oxidative stress.

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RADIATION RESEARCH 129, 333–344 (1992)

Radiation-Induced DNA Damage as a Function of Hydration

I. Release of Unaltered Bases

STEVEN G. SWARTS,* MICHAEL D. SEVILLA,† DAVID BECKER,† CHRISTOPHER J. TOKAR,* AND KENNETH T. WHEELER*

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SWARTS, S. G., SEVILLA, M. D., BECKER, D., TOKAR, C. J., AND WHEELER, K. T. Radiation-Induced DNA Damage as a Function of Hydration. I. Release of Unaltered Bases. *Radiat. Res.* 129, 333–344 (1992).

The release of unaltered bases from irradiated DNA, hydrated between 2.5 and 32.7 mol of water per mole of nucleotide (Γ), was investigated using HPLC. The objective of this study was to elucidate the yield of the four DNA bases as a function of dose, extent of hydration, and the presence or absence of oxygen. The increase in the yield of radiation-induced free bases was linear with dose up to 90 kGy, except for the DNA with $\Gamma = 2.5$, for which the increase was linear only to 10 kGy. The yield of free bases as a function of Γ was not constant in either the absence or the presence of oxygen over the range of hydration examined. For DNA with Γ between 2.5 and 15, the yield of free bases was nearly constant under nitrogen, but decreased under oxygen. However, for DNA with $\Gamma > 15$, the yield increased rapidly under both nitrogen and oxygen. The yield of free bases was described by a model that depended on two factors: 1) a change in the DNA conformation from a mixture of the A and C conformers in vacuum-dried DNA to predominantly the B conformer in the fully hydrated DNA, and 2) the proximity of the water molecules to the DNA. Irradiation of the inner water molecules ($\Gamma < 15$) was less efficient than irradiation of the outer water molecules ($\Gamma > 15$), by a factor of ≈ 3.3, in forming DNA lesions that resulted in the release of an unaltered base. This factor is similar to the previously published relative efficiency of 2.8 with which hydroxyl radicals and base cations induce DNA strand breaks. Our irradiation results are consistent with the hypothesis that the *G* value for the first 12–15 water molecules of the DNA hydration layer is the same as the *G* value for the form of DNA to which it is bound (i.e., the pseudo-C or the B form). Thus we suggest that the release of bases originating from irradiation of the hydration water is obtained predominantly: (1) by charge transfer from the direct ionization of the first 12–15 water molecules of the primary hydration layer and (2) by the attack of hydroxyl radicals generated in the outer, more loosely bound water molecules. © 1992 Academic Press, Inc.

INTRODUCTION

The DNA molecule is considered to be the most important target for the cellular effects of ionizing radiation. As such, much attention has been devoted to elucidating the mechanisms of radiation action on DNA including the formation of DNA lesions, the removal of these lesions through various repair processes, and the biological effects of the residual unrepaired or misrepaired lesions. Of particular interest are the physico–chemical events that occur upon irradiation of the DNA molecule. An early effect of radiation is the formation of free radicals on the DNA, either through the direct ionization of the DNA molecule or through an indirect mode via reaction of the DNA with radicals formed in the surrounding environment (e.g., $OH\cdot$, e^-_{aq}, and $H\cdot$). Moreover, the types of DNA radicals produced and the mechanisms by which they form nonradical DNA lesions may depend on the relative contributions of the direct and indirect effects.

One factor that may contribute significantly to these processes is the effect of the water of hydration on the formation and reaction of the radiation-induced radicals in and/or around the DNA. These water molecules are coordinated to the DNA to varying degrees that depend on their location within the DNA hydration shell, and have properties that are different from those found for free water as measured by IR, NMR, and other techniques (*1–14*). The primary hydration layer consists of 20–21 water molecules per nucleotide, 12–15 of which are bound tightly to the DNA (*3, 10, 14*). The tightly bound water molecules are impermeable to cations (*8*) and do not form an ice-like structure when cooled below 0°C (*9*). Further characterization of these water molecules shows them to be less mobile than free water, but more mobile than ice water (*10, 15*). Another 6–9 water molecules per nucleotide surround the inner 12–15 water molecules (*14*). These water molecules complete the primary hydration layer and are distinct from bulk water as demonstrated by their different infrared spectral characteristics (*16*).

The unique properties of the water of hydration appear to play a major role in the physico–chemical events that occur in the DNA. For example, exposure of DNA to UV radiation altered the type and quantity of DNA damage produced as the hydration of the DNA was increased (*17*). Also, the sensitivity of a diploid yeast to UV radiation *in*

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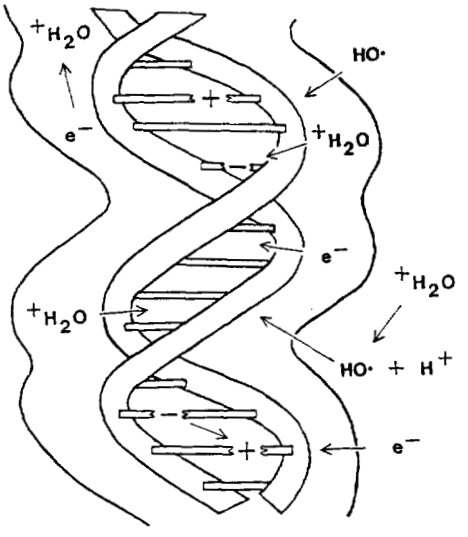

FIG. 1. A schematic diagram of the radicals produced by ionizing radiation in both the DNA molecule and the two regions of hydration, i.e., the inner 15 water molecules that are more tightly bound to the DNA and all outer water molecules that are more loosely bound.

vitro depended on the water vapor pressure in the rehydration environment (*18*). In both cases, the effects were attributed to changes in the DNA induced by varying the levels of hydration. However, with regard to exposure to ionizing radiation, it is not known how radicals formed in these hydration layers can affect the DNA, especially *in vivo*.

One important step in comprehending the *in vivo* mechanisms for induction of radiation damage in DNA involves elucidating how the various hydration layers affect the types and quantities of DNA damage. Figure 1 shows a schematic diagram of the radicals produced by ionizing radiation in both the DNA and the two regions of hydration. Several investigators have posed the following question (*5*, *19–21*). For the water molecules that are closely associated with the DNA (Fig. 1), is the DNA damage produced via charge transfer from the initial radicals formed in the water (H_2O^+ and e^-_{dry}), or via reactions with bulk water radicals (OH · and e^-_{aq})? Results from the study of dry and hydrated DNA using pulse radiolysis/luminescence techniques suggest that hydration plays a significant role in modifying the DNA radical species, through either changes in the DNA conformation or changes in the types of water radicals acting on the DNA (*22*, *23*). Changes in the level of DNA hydration have also been shown to affect the mobility of radiation-induced electrons in the hydration water (*24*). In addition, ESR studies have shown that at low temperatures direct irradiation of hydrated DNA results in ion radicals on the DNA bases with little evidence for deoxyribose/phosphate radical formation (*25*). These radicals may result in the formation of nonradical DNA lesions that are different from those lesions produced by the radicals formed by irradiating bulk water. The influence of the hydration layer on the formation of radiation-induced DNA lesions has also been studied using solid-state DNA models (*26–28*). From these studies, there was evidence of an effect of the hydration layer on some types of radiation-induced DNA damage. For example, the yield of DNA strand breaks and DNA–DNA crosslinks depended on the level of hydration (*26*, *28*). However, few or no data exist on the hydration dependence of several other important DNA lesions such as the release of unaltered bases,[1] specific DNA base damage, or sugar damage.

To extend our understanding of the influence of the hydration water on the types and quantities of radiation-induced DNA damage, we have analyzed several different types of lesions produced by ionizing radiation in DNA that has been hydrated to various levels. These types of DNA lesions include the release of unaltered bases, specific DNA base damage, and nucleoside damage. The work presented here examines the amount of radiation-induced release of unaltered DNA bases as a function of dose, extent of hydration, and the presence or absence of oxygen.

MATERIALS AND METHODS

Preparation of the DNA for hydration. Approximately 20 mg of dry salmon sperm DNA (Sigma, type III, sodium salt) was transferred to acid-washed vials (Supelco 3 dram, screw cap). The DNA was assayed for iron and copper content by atomic absorption spectroscopy and found to have 1 iron per 6,200 nucleotides and 1 copper per 140,000 nucleotides. Both the empty vials and the vials containing DNA were evacuated for 7 to 9 days using an in-line liquid N_2 trap until a constant weight of the DNA was obtained. The vials were removed under a slow stream of N_2 gas. Upon removal from the vial, the dried DNA was weighed immediately and then dissolved in 1.0 ml of deionized water in the original drying vial. After storage for 2 days in the dark at 4°C, the resultant DNA/water gels were frozen and lyophilized for 7 days along with empty control vials using a liquid N_2 trap. This procedure produced a dry homogeneous DNA plug that had a volume of ≈ 1 cm^3. After the DNA was freeze-dried, the control and experimental vials were removed from the chambers under a stream of N_2 gas and weighed immediately.

Hydration of the DNA. Control and experimental vials were placed into hypoxia chambers (Controls Katharobic, Philadelphia, PA) containing either N_2 (<10 ppm O_2) or O_2 gas at 1 atm. The humidity in each chamber was controlled using dishes containing various saturated salt solutions (100% relative humidity, deionized water; 84% relative humidity, saturated KCl; 58% relative humidity, saturated NaBr; 43% relative humidity, saturated K_2CO_3; 0% relative humidity, dry N_2 or O_2 gas) (*26*, *29–32*). After sealing, the contents of the chambers were subjected to five repetitions of evacuation to $\approx 5 \times 10^{-4}$ atm, followed by flushing with either N_2 or O_2 gas to 1 atm. Each chamber was equipped with a barrier to prevent splattering of salt solutions onto control and experimental vials during the evacuation/re-gas cycling. After holding the chambers for 7 days at 20°C, both the control and experimental vials were removed from the chambers, sealed under a slow stream of either N_2 or O_2, and weighed immediately.

Irradiation of the hydrated DNA. Irradiation of the hydrated DNA was performed using a ^{137}Cs irradiator (J. L. Shepherd and Associates, Inc.)

[1] I. Krönke, Co-γ-bestrahlung von fester DNA und isolierten zellkernen aus hühnererythrozyten. Ph.D. dissertation. Ruhr-Universität, Bochum, 1983.

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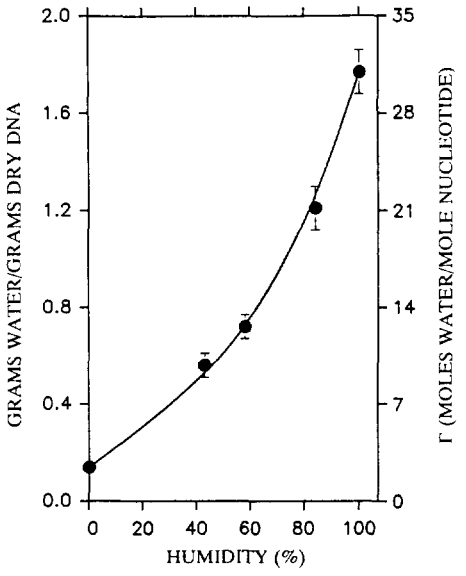

FIG. 2. Hydration of double-stranded salmon sperm DNA as a function of the relative humidity in the hydration chambers. Hydration is given either as the number of grams of water per gram of dry DNA or as the number of water molecules per nucleotide (Γ). The points represent the mean ± 1 SEM of at least six independent experiments.

with a dose rate of 41–51 Gy/min, depending on the position of the samples within the jig. Dose rates were calculated from both Fricke and ionization chamber measurements, which differed by <3%.

Isolation of the released bases. After irradiation, the DNA samples were stored in the dark at 20°C for 5 days. Each DNA sample was then dissolved in 2 ml of N_2- or O_2-saturated 10 m*M* ammonium acetate buffer (pH 7) and stored in the dark for 3 days at 4°C. The DNA solutions were transferred to centrifuge tubes and 8.2 ml of cold absolute ethanol was added. After holding in the dark overnight at 4°C to precipitate the DNA fully, each sample was centrifuged at 39,000 rpm in a Beckman SW-41 Ti rotor for 2 h. The supernatant was decanted from the DNA pellet, partially dried by evaporation under N_2 in a 40°C water bath, and then lyophilized.

Analysis of the released bases. Depending on the dose, all lyophilized supernatants were redissolved in 1–4 ml of deionized water and vortexed for 1 min. Unaltered bases were separated by HPLC using a Beckman Ultrasphere ODS column (4.6 × 250 mm) with a gradient of 0.01 m*M* ammonium phosphate, pH 4.5 (buffer A), and 20% acetonitrile in deionized water (buffer B) at a flow rate of 1 ml/min. The bases were measured at 260 nm using a Beckman 166 UV/VIS detector, and were quantified by both internal and external standardization methods using authentic compounds. Data analysis was performed on a Beckman System Gold computer workstation. Statistical analysis of the data was performed using the SAS statistical package on a VAX 750/11 computer.

Assay for total base release. To determine if all of the radiation-induced release of DNA bases had been obtained in the workup procedure, ≈1 mg of each DNA pellet was dissolved in 2 ml of 0.15 *M* ammonium acetate buffer (pH 7) and heated to 37°C for 24 h. After cooling to 4°C, cold anhydrous ethanol was added to the samples to yield a 70% ethanol solution. The DNA was stored overnight at 4°C in the dark and then assayed exactly as described above.

RESULTS

The hydration of salmon sperm DNA increased as a function of the relative humidity, as shown in Fig. 2. The extent of the hydration, as expressed by the number of moles of water per mole of nucleotide (Γ), was as follows: 100% relative humidity, 32.7; 84%, 22.4; 58%, 13.2; 43%, 10.3; and 0%, 2.5. The Γ value of 2.5 for the 0% samples is the tightly bound hydration waters attached to the sodium phosphate group that cannot be removed via vacuum desiccation of the DNA samples (*10*). The values for the extent of hydration of the DNA are consistent with those reported previously (*26*, *30*) and correspond to filling the DNA hydration layers as follows (*14*): (1) the 43 and 58% relative humidity values of 10.3 and 13.2 are the approximate numbers of water molecules that are tightly bound to the DNA, (2) the 84% value of 22.4 is the approximate number of water molecules required to complete the primary hydration layer (Γ = 20–21), and (3) the additional water molecules at 100% relative humidity are the more loosely bound water molecules in the secondary hydration layer.

The chromatogram in Fig. 3 was obtained from the resuspended supernatant of the DNA that had been hydrated to Γ ≈ 22 and irradiated to a dose of 90 kGy. The gradient and buffers that were selected provided a good and fairly rapid separation of the four bases with only a slight variation (<0.1 min) in their retention times from run to run. The release of several altered bases was demonstrated by the presence of peaks at 5.8, 10.4, and 18.3 min. These altered bases were identified as 5-hydroxymethyluracil, 8-hydroxyguanine, and 8-hydroxyadenine, respectively. This release of altered bases is consistent with the release of altered thymine from DNA irradiated in aqueous solution reported previously (*33*).

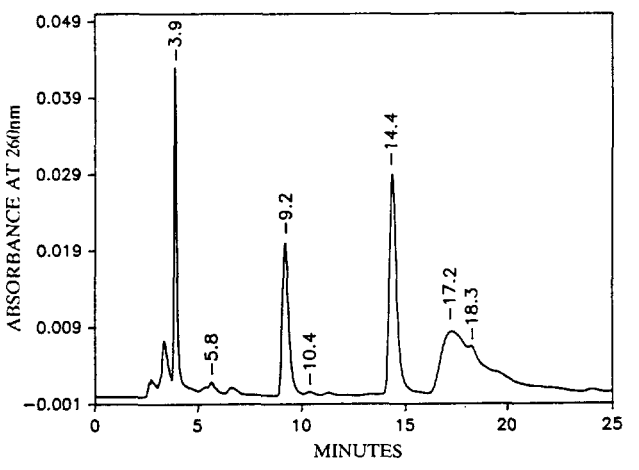

FIG. 3. HPLC chromatogram of the bases released from DNA that had been hydrated to ≈22 water molecules per nucleotide and irradiated with 90 kGy under N_2. The chromatogram was obtained at a flow rate of 1 ml/min with the following gradient profile: 100% A, 3 min; 0–12.8% B in A, 20 min; 12.8–100% B in A, 7 min; 0–100% A in B, 10 min; 100% A, 8 min. The major peaks at retention times of 3.9, 9.2, 14.4, and 17.2 min were identified as cytosine, guanine, thymine, and adenine, respectively. Three minor peaks at 5.8, 10.4, and 18.3 min were identified as 5-hydroxymethyluracil, 8-hydroxyguanine, and 8-hydroxyadenine, respectively.

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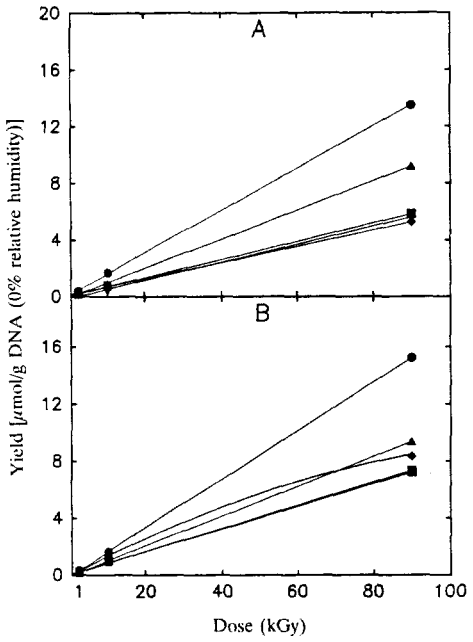

FIG. 4. The average yield of unaltered bases from double-stranded salmon sperm DNA as a function of both dose and the degree of hydration in the absence (A) and presence (B) of O_2. The data were calculated using the weight of vacuum-dried DNA ($\Gamma = 2.5$) to emphasize the contribution of radiation-induced water radicals to the release of bases. The points represent the mean of two or three independent experiments. The standard deviation for each point was 5–15% of the value and has been omitted for clarity. Relative humidity: (●) 100%, (▲) 84%, (■) 58%, (▼) 43%, (♦) 0%.

With the exception of "dry" DNA ($\Gamma = 2.5$), the release of all four unaltered bases from double-stranded salmon sperm DNA irradiated under N_2 (Fig. 4A) or O_2 (Fig. 4B) was a linear function of dose to 90 kGy. At 0% relative humidity, the yield of free bases from DNA irradiated under both N_2 and O_2 clearly exhibited a curvilinear function as the dose exceeded 10 kGy. The DNA irradiated under O_2 showed a more pronounced nonlinear dose response than the DNA irradiated under N_2. These results are consistent with the expectation that long-lived radicals in dry DNA (*32*) will lead either to a higher number of recombinations or to second-hit events.

The dose–response functions in Figs. 4A and 4B also demonstrated a marked change in slope as Γ was increased. Because the yields were calculated from the prehydration weight of the DNA, any additional base release outside of that formed from direct ionization of the DNA will be reflected as a change in the slope. The change in the slope was not directly proportional to the number of added water molecules, as was illustrated by the greater increase in the slopes for the DNA hydrated at 84 and 100% relative humidity. This was demonstrated more clearly in a plot of the average yield for the combined base release as a function of Γ for DNA irradiated with either 2 or 90 kGy under N_2 (Fig. 5). At 90 kGy, the yield of released bases as a function of Γ was essentially constant at 0.05 μmol/g/Γ for $\Gamma < 15$, but abruptly increased to 0.48 μmol/g/Γ for $\Gamma > 15$ (Fig. 5A). Although the yield per kilogray was slightly less at 90 kGy, the shape of the yield curve as a function of the Γ (yield profile) was similar at both 2 and 90 kGy (Fig. 5).

A plot of the average yield of released bases as a function of Γ for DNA irradiated under O_2 resulted in different profiles at 2 and 90 kGy (Fig. 6). The value for DNA with $\Gamma = 2.5$ was significantly higher under O_2 (9.5 ± 0.7 μmol/g DNA at 90 kGy) than was the value found under N_2 (6.0 ± 0.3 μmol/g DNA at 90 kGy). As Γ increased, the yield under O_2 actually decreased to $\approx$8 μmol/g DNA at $\Gamma \approx 15$. For $\Gamma > 15$, the yield again increased at approximately the same rate as that found for DNA irradiated under N_2; at $\Gamma = 32.7$, the yield was 14.4 ± 0.2 μmol/g DNA for DNA irradiated under O_2 (Fig. 6A) compared to 15.3 ± 0.1 μmol/g DNA for DNA irradiated under N_2 (Fig. 5A). As noted in the N_2 data (Fig. 5), the yield profile for DNA irradiated under O_2 was similar at 2 and 90 kGy, except that the initial decrease in the average yield of unaltered bases for $\Gamma < 15$ was more pronounced at the 2-kGy dose (Fig. 6B).

An examination of the individual *G* values calculated from the posthydration weight of the DNA for the four unaltered bases from double-stranded DNA irradiated under N_2 and O_2 showed that all bases were not released to the same extent (Tables I, II). The *G* values for the release of

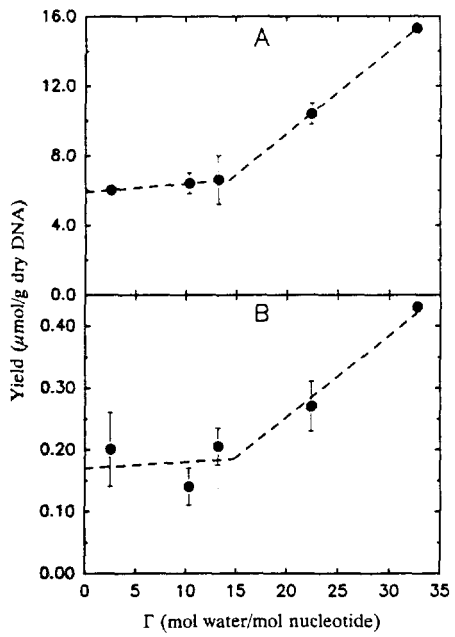

FIG. 5. The average yield of unaltered bases as a function of the moles of water per mole of nucleotide (Γ). The DNA was irradiated under N_2 with a dose of 90 kGy (A) or 2 kGy (B). The yields were based on the dry weight of the DNA ($\Gamma = 0$). Each point represents the mean ± 1 SD of two or three independent experiments. The straight portions of the dashed curves were fitted by a linear regression of the appropriate individual experiment data.

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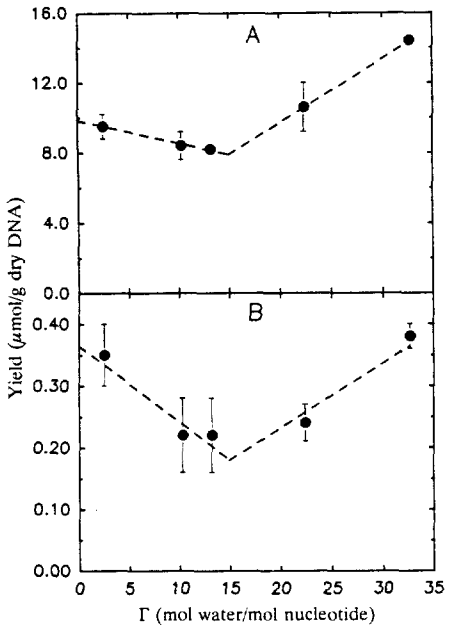

FIG. 6. The average yield of unaltered bases as a function of the moles of water per mole of nucleotide (Γ). The DNA was irradiated under O_2 with a dose of 90 kGy (A) or 2 kGy (B). The yields were based on the dry weight of the DNA (Γ = 0). Each point represents the mean ± 1 SD of two or three independent experiments. The straight portions of the dashed curve were fitted by a linear regression of the appropriate individual experiment data.

adenine, cytosine, and thymine as a function of increasing Γ followed the trend observed in the combined base release where G value maxima were noted at Γ = 2.5 and 32.7 (Tables I, II). However, the G values for guanine were significantly ($P < 10^{-3}$) less than those for the other three bases at all the hydration levels. The maximum G value for the release of guanine occurred at Γ = 2.5; the remaining G values were similar with no obvious response pattern (Tables I, II).

The yields for the release of individual bases, calculated using the weight of dry DNA (Γ = 0), were plotted as a function of Γ for DNA irradiated with a dose of 90 kGy under N_2 or O_2 (Fig. 7). As observed for the combined base release, two functions described the release of each base: one for Γ < 15 and one for Γ > 15. As would be expected based on the posthydration G values, the yield profile for guanine was lower in comparison to the other three bases. Under N_2, it appeared that the yields for adenine, cytosine, and thymine for Γ < 15 were similar, but diverged at Γ > 15, with thymine having the greatest slope, followed by adenine, then cytosine (Fig. 7A). Under O_2, the yields of these three bases were again similar for Γ < 15 (Fig. 7B). However, the yields of these three bases did not appear to diverge at Γ > 15, although a slight divergence could have been masked by the scatter in the data (Fig. 7B).

One problem that could affect the analysis of the base-release data is the low solubility of some of the bases, especially guanine (*34*). To determine the magnitude of this problem, a known amount of each individual base approximately equal to that obtained in the radiation experiment was added to a series of experimental samples, and the loss of the added bases during sample processing was determined. Greater than 98% of each added base, including guanine, was recovered. Consequently, our base-release data are not affected by recovery problems.

As noted in the literature, there is the possibility that all of the radiation-induced base release may not be expressed under certain conditions (*35–39*). To obtain the total radiation-induced release of bases, the DNA must be heated for a period of time, generally at 37°C for 24 h (*35*). When this procedure was performed, the additional release of pyrimidines was greater than the release of purines (Table III). For the pyrimidines, more thymine was released than cytosine (Table III). The amount of additional bases released did not vary as a function of either Γ or the absence or presence of oxygen. If the additional bases released after heating were

TABLE I

G Values for the Release of Unaltered Bases from DNA Irradiated under N_2 as a Function of the Degree of Hydration[a]

Humidity (%)	Cytosine	Guanine	Thymine	Adenine	Combined
100	0.015 (0.001)[b]	0.0094 (0.0006)	0.020 (0.002)	0.017 (0.001)	0.061 (0.002)
84	0.013 (0.001)	0.0095 (0.0009)	0.017 (0.001)	0.016 (0.002)	0.055 (0.006)
58	0.013 (0.002)	0.0080 (0.0001)	0.013 (0.001)	0.014 (0.002)	0.048 (0.007)
43	0.014 (0.001)	0.0079 (0.0002)	0.012 (0.001)	0.013 (0.001)	0.047 (0.003)
0	0.021 (0.001)	0.0120 (0.0004)	0.019 (0.001)	0.021 (0.001)	0.073 (0.003)

[a] *G* values were derived from the slopes obtained using a linear regression analysis of the base release yields (μmol/kg DNA) as a function of four doses (1, 2, 10, and 90 kGy) for DNA hydrated under 43–100% relative humidity. For DNA hydrated under 0% relative humidity, only the three lower doses (1, 2, and 10 kGy) were used in the *G*-value calculations due to a nonlinearity in the response for the 90-kGy data. The posthydration weight of the DNA was used in the *G*-value calculations. *G* values are expressed as micromoles/joule.

[b] Mean ± 1 SD of two or three independent experiments.

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TABLE II

G Values for the Release of Unaltered Bases from DNA Irradiated Under O_2 as a Function of the Degree of Hydration[a]

Humidity (%)	Cytosine	Guanine	Thymine	Adenine	Combined
100	0.019 (0.001)[b]	0.0060 (0.0005)	0.023 (0.002)	0.019 (0.001)	0.067 (0.004)
84	0.014 (0.001)	0.0064 (0.0003)	0.017 (0.001)	0.017 (0.001)	0.054 (0.004)
58	0.013 (0.001)	0.0079 (0.0003)	0.012 (0.002)	0.016 (0.001)	0.049 (0.004)
43	0.015 (0.001)	0.0088 (0.0004)	0.013 (0.001)	0.017 (0.001)	0.054 (0.003)
0	0.040 (0.001)	0.0250 (0.0006)	0.036 (0.001)	0.039 (0.001)	0.140 (0.004)

[a] *G* values were derived from the slopes obtained using a linear regression analysis of the base release yields (μmol/kg DNA) as a function of four doses (1, 2, 10, and 90 kGy) for DNA hydrated under 43–100% relative humidity. For DNA hydrated under 0% relative humidity, only the three lower doses (1, 2, and 10 kGy) were used in the *G*-value calculations due to a nonlinearity in the response for the 90-kGy data. The posthydration weight of the DNA was used in the *G*-value calculations. *G* values are expressed as micromoles/joule.

[b] Mean ± 1 SD of two or three independent experiments.

added to the data in Fig. 7A, the separation of the purine and pyrimidine curves increased, but the shape of the curves and the slopes remained the same (data not shown).

DISCUSSION

The release of unaltered bases from γ-irradiated solid-state DNA depends on the degree to which the DNA is hydrated (Figs. 4–6). The incremental amount of base release observed as water molecules are added to the DNA varies depending on the location of the water relative to the DNA. The large difference in base release for the first ≈15 added water molecules relative to the last ≈18 water molecules (Figs. 5 and 6) suggests that there is a fundamental difference between the inner and outer water of hydration. Interestingly, this transition seems to occur at the interface between the inner 12–15 tightly bound water molecules and the outer 6–9 more loosely bound water molecules that constitute the primary hydration layer (*3, 10, 14*). In addition, changes in the conformational properties of the DNA may be imparted by changing the degree of hydration (*14, 16, 40*). These conformational changes could also be responsible for some of the results in Figs. 5 and 6.

By its proximity to the DNA, the inner water of hydration has the ability to interact directly with the DNA. Charged species (H_2O^+, e^-_{dry}) that are initially formed by irradiation of the inner water molecules of the primary hydration layer may be transferred directly to the DNA (*19, 20, 41, 42*, Fig. 1). For the irradiated water molecules that

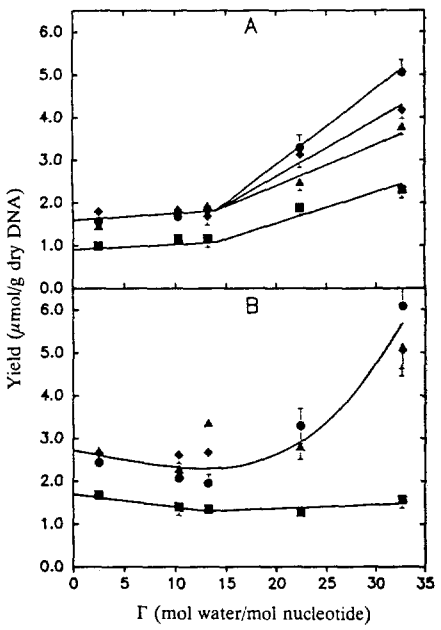

FIG. 7. The average yield of the individual bases as a function of Γ for DNA receiving a dose of 90 kGy in the absence (A) or presence (B) of O_2. The yields were based on the dry weight of the DNA (Γ = 0). Each point represents the mean ± 1 SD of two or three independent experiments. The straight portions of the curves were fitted by a linear regression analysis of the appropriate individual experiment data. The nonlinear curve in B was fitted by eye. (♦) adenine, (▲) cytosine, (■) guanine, (●) thymine.

TABLE III

The Effect of Heating for 24 h at 37°C on the Release of the Individual Bases from Double-Stranded Salmon Sperm DNA Irradiated with 90 kGy under either Nitrogen or Oxygen

Base	Additional base release (μmol/g of DNA)[a]
Cytosine	0.49 ± 0.08[b]
Guanine	IN[c]
Thymine	0.64 ± 0.19
Adenine	IN[c]

[a] Calculated using the weight of vacuum-dried DNA (Γ = 2.5) and corrected for spontaneous base release due to heating. The values in the table are expressed as micromoles per gram of DNA.

[b] Values are the mean ± 1 SD of all five hydrations under both N_2 and O_2 from two independent experiments.

[c] Indeterminate; the values were not statistically different ($P > 0.05$) from the values for spontaneous base release due to heating.

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are removed further from the DNA, it is expected that the hydroxyl radical will be the predominant damaging species (*20, 43*, Fig. 1). From pulse radiolysis and laser-induced photoionization studies, the hydroxyl radical has been found to be more efficient at causing strand breaks than charged radicals on the bases (*43–45*). Consequently, a significant change in the yield of unaltered bases should occur where the mechanism for inducing strand breaks shifts from predominantly charged species transfer to predominantly hydroxyl radical attack, although other mechanisms could play a role (*46, 47*). Such a change in the yield of unaltered bases was observed when 12–15 water molecules were added to the DNA (Figs. 5–7).

Both the conformation and the mobility of the DNA demonstrate significant changes with increasing DNA hydration (*14*). With respect to conformation, the changes observed in the DNA can be best described by a conversion from a pseudo-C conformation (a mixture of the A and C conformers) in the "dry" state ($\Gamma = 2.5$) to a B conformation, as the DNA is hydrated to $\Gamma = 20$–21 (*12, 13, 16, 40*). Accompanying this conformation change is an alteration in the rigidity of the DNA, where the DNA behaves like a crystalline solid for DNA containing less than 10% water, but rapidly becomes a highly viscous solution for DNA containing greater than 50% water (*4*). In addition, the amplitude of purine motion, the restricted rotation about the tilted axis, and the rotational diffusion have been shown to increase with increasing values of Γ (*6, 7*). The conversion of the DNA from the pseudo-C conformation to the B conformation results in a narrowing of the major groove (*14*) and may limit the accessibility of oxygen to DNA base radicals. In addition, the pseudo-C to B conformational change will stabilize the base stacking interactions (*22*), which likely leads to an increase in the mobility through the stacked bases of the dry electron and the positive hole formed by the direct irradiation of DNA (*48–50*). This can result in a decrease in the base ion radicals because of an increase in charge recombination (Fig. 1). Since it has been shown that DNA base ion radicals can produce strand breaks by formation of a sugar radical via H-abstraction from a neighboring sugar residue (*44, 51–60*), a decrease in base radicals should result in lowered yields of the released bases.

In an attempt to better understand the effects of the water of hydration on the release of unaltered bases after irradiation, we have developed a model that describes the contributions of: 1) the damage resulting from direct ionization of the DNA, in both the pseudo-C form and the B-form, and 2) the damage resulting from ionization of the water of hydration. Two types of water molecules are assumed to be present. Water molecules of the first type (W1) are closer to the DNA and water molecules of the second type (W2) are further away from the DNA. Γ_{W1} is the number of water molecules of the first type and is treated as a parameter to be fitted to the data. The fraction of the DNA in the B-form (X_B) is determined by adjusting the results of Wolf and Hanlon (*16*) to $\Gamma = 2.5$ at 0% relative humidity (*10*). This gives

$$X_B = 0.0563\,\Gamma - 0.1407 \quad (\Gamma = 0 \text{ to } \Gamma = 14.7)$$

$$X_B = 0.0076\,\Gamma + 0.575 \quad (\Gamma > 14.7),$$

where Γ is the number of water molecules per nucleotide of DNA (sodium salt). The total yield of released, unaltered bases is then given by

$$Y(\text{total}) = G(\text{C})X_C M_{DNA} + G(\text{B})X_B M_{DNA} + G(\text{W1})M_{W1} + G(\text{W2})M_{W2}, \quad (1)$$

where Y(total) is the total base release expressed as μmol/g/kGy of dry DNA ($\Gamma = 0$); G(C) and G(B) are the G values for unaltered bases released by direct ionization of the pseudo-C form and B-form of the DNA, respectively; X_C and X_B are the mole fractions of the DNA in the pseudo-C form and B-form, respectively; and M_{DNA} is the total mass of the DNA. Since these results are expressed on a per gram basis, $M_{DNA} = 1$. We also assume that $X_C + X_B = 1$; that is, no other conformation of the DNA is present. Finally, G(W1) and G(W2) are the G values for the two types of water, and M_{W1} and M_{W2} are the masses of the two types of water.

A multiple regression fit of Eq. (1) to the 13 individual data points for DNA samples irradiated with 90 kGy under N_2 or O_2 was performed at various values of Γ_{W1} (Figs. 5 and 6). Valid solutions to Eq. (1) require that all G values be positive. Positive values for G(B) and G(W1) were obtained only at Γ_{W1} values of 14.4–14.6 for DNA irradiated in an N_2 atmosphere and 15.0–15.5 for DNA irradiated in an O_2 atmosphere. For Γ_{W1} values where positive solutions were found, G(C) and G(W2) were essentially constant, whereas G(B) and G(W1) varied inversely, with the sum of G(B) and G(W1) being a constant.

Neither G(B) nor G(W1) can have trivially small values (e.g., <0.015 μmol/J). Therefore, if the sum of G(B) and G(W1) is 0.7–0.8 (Table IV), then G(B) and G(W1) must be roughly equal in value. When the sum of G(B) and G(W1) was partitioned equally between the two parameters, Γ_{W1} was 14.5 for DNA irradiated under N_2 and 15.2 for DNA irradiated under O_2. Table IV presents the best estimates of the various parameters derived from the multiple regression fit with the sum of G(B) and G(W1) partitioned equally between them. Both fitted Γ_{W1} values are consistent with the 12–15 water molecules per nucleotide that have been reported to be bound tightly to the DNA (*3, 10, 14*). In addition, the similarity between G(B) and G(W1) suggests that the base release which results from irradiation of the W1 water is mechanistically related to that formed from direct ionization of the DNA.

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TABLE IV
Component G Values for Total Base Release from Irradiated DNA[a–c]

Parameter	N_2	O_2
G(C)	0.06 ± 0.01[d]	0.10 ± 0.01
G(B) + G(W1)	0.074 ± 0.01[c]	0.077 ± 0.01[c]
G(W2)	0.10 ± 0.02	0.09 ± 0.025
Γ_{W1}	14.5[e]	15.2[e]

[a] G values are expressed in micromoles/joule and are derived from a multiple regression fit of Eq. 1 to the 90-kGy data in Figs. 5 and 6. The component G values are for the DNA pseudo-C form, DNA B form, first water type (W1), and second water type (W2). The data obtained at lower doses have greater uncertainties; however, they suggest that the value for G(C), in both N_2 and O_2, increased about 25% over that reported in the Table.

[b] All G values were positive only for a small range (ca. ± 0.2) of Γ_{W1} (see text). G(C) and G(W2) varied less than 10% for all positive values of Γ_{W1}.

[c] Although the individual values varied inversely, the sum of G(B) and G(W1) was constant for all values of Γ_{W1} for which G(B) and G(W1) were positive. For the reported Γ_{W1} values, the sum was apportioned equally between G(B) and G(W1).

[d] Error limits for the G values were chosen from the largest of (1) an approximation of the standard error of the parameter estimate from the multiple regression analysis, or (2) the range of G values obtained subject to the conditions that all G values were positive and that neither G(B) nor G(W1) became unreasonably small (<0.015 μmol/J).

[e] The error associated with Γ_{W1} was estimated at ± 2–3 water molecules/nucleotide.

To get a better estimate of the G(B) and G(W1) values, a second equation was developed in which the type W1 water was presumed to only increase the target mass of the DNA, as suggested from the results of Eq. (1). The total yield of released, unaltered bases can then be expressed as

$$Y(\text{total}) = G'(\text{C})(X_{\text{C}})(M_{\text{DNA}} + M_{\text{W1}}) + G'(\text{B})(X_{\text{B}})(M_{\text{DNA}} + M_{\text{W1}}) + G'(\text{W2})M_{\text{W2}}, \quad (2)$$

where the G' values are the new estimates based on the mass of the pseudo-C DNA and B DNA, plus the mass of the W1 water associated with each DNA form.

The best multiple regression fit of Eq. (2) to the 13 individual N_2 data points in Fig. 5A gave the following G' values in micromoles/joule: G'(C) = 0.059 ± 0.01, G'(B) = 0.029 ± 0.01, G'(W2) = 0.10 ± 0.02. This unique solution to Eq. (2) was obtained at Γ_{W1} = 12.8, a value that agrees with the 12–15 water molecules per nucleotide that are reported to be tightly bound to the DNA (*3, 10, 14*). G'(C) and G'(W2) were identical to G(C) and G(W2) determined from the best fit to Eq. (1) (Table IV). By subtracting the uniquely determined value of G'(B) from the sum of G(B) and G(W1) in Table IV, a G(W1) value of 0.045 ± 0.01 μmol/J was obtained. Thus the G value for the W1 water (0.045 μmol/J) was halfway between the G value for pseudo-C DNA (0.06 μmol/J) and the G value for B DNA (0.029 μmol/J). Although more variable due to the scatter in the data, the fit of Eq. (2) to the O_2 data in Fig. 6A generated conclusions identical to those derived from the N_2 data. An excellent fit to the 90-kGy N_2 (Fig. 8A) and O_2 (Fig. 8B) data was obtained when the unique value of G(B) derived from Eq. (2) was used to get a unique value of G(W1). Taken together, these results suggest that the G value for the W1 water is the same as the G value for the particular conformation of the DNA to which they are bound. Thus the data support the concept that the predominant mechanism by which ionization of W1 water causes the release of unaltered bases from the DNA is similar to the mechanism by which direct ionization of the DNA causes the release of unaltered bases, i.e., by dry charge transfer rather than by hydroxyl radical attack.

Our result, G(C) > G(B), is consistent with the change in base stacking that occurs with this change in conformation (Table IV). The increased base stacking in the B conformation permits greater transport through the bases of the electron and hole that are formed by the radiation (*48–50*). This, in turn, leads to more charge recombination in the B form, and a concomitant lower G value for base release.

Type W2 water is associated with, but not directly bound to, B DNA. The G value for base release resulting from the ionization of this water is 0.10 μmol/J, which is larger by a

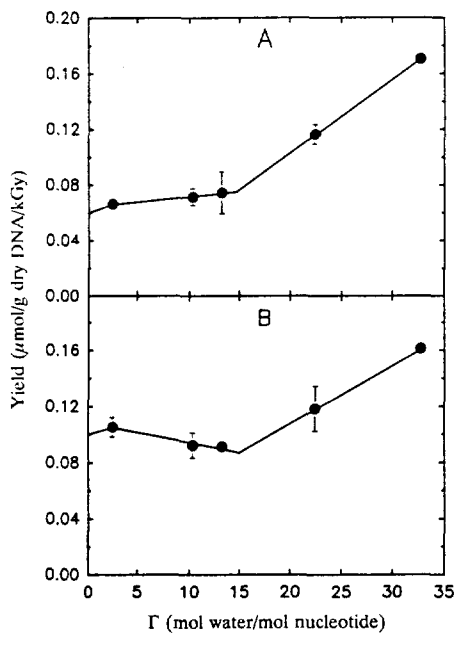

FIG. 8. The average yield of unaltered bases as a function of Γ for DNA receiving a dose of 90 kGy in the absence (A) or presence (B) of O_2. The yields were calculated on the basis of the dry weight of DNA (Γ = 0). The data points come from Fig. 5A (A) and Fig. 6A (B). The line is the multiple regression fit of Eq. (1) using the G(B) value obtained from Eq. (2). The correlation coefficients for the lines in the figure were 0.96 for N_2 and 0.80 for O_2.

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factor of about 3.3 than the G value estimated for the more closely bound water molecules (0.029 μmol/J). The G(W2)/$G(W1)_B$ ratio of 3.3 is close to the value of 2.8 found for the efficiency of strand-break production by hydroxyl radicals relative to the efficiency of strand-break production by cation base radicals (*43, 45, 50*). This agreement leads us to believe that irradiation of type W2 water causes base release largely through hydroxyl radical attack (Fig. 1). The value for G(W2) is approximately twice that found for DNA in dilute aqueous solution (*27, 35, 38*). This suggests that the DNA is scavenging most of the hydroxyl radicals formed from the type W2 water, probably due to these outer water molecules being less than 8 Å away from the surface of the DNA. Therefore, most OH radicals will react with the DNA. In dilute solution, both geometrical considerations and the recombination of hydroxyl radicals that form in spurs lower the effective yield of hydroxyl radicals that can react with the DNA.

Hydrogen peroxide is one radiation-induced product that could mimic hydroxyl radical reactions on DNA (*46, 61*). This product can be formed in high yields (G = 1.7 to 3.5 events per 100 eV) in irradiated aqueous solutions (*39, 62*). Hydrogen peroxide has been shown to induce DNA strand breaks (*63, 64*), specific base damage (*47, 61, 64*), and base release (*39, 47, 63*) in unirradiated DNA in aqueous solution. Thus hydrogen peroxide formed in the hydrated DNA systems might play a role in the yield of released bases for $\Gamma > 15$. However, the formation of hydrogen peroxide from the recombination of two hydroxyl radicals is less likely to occur in a monolayer of water on the DNA than in aqueous solutions of DNA due to geometrical considerations. Also, there is very little iron and copper in our hydrated DNA to support the Fenton chemistry required to cause the release of unaltered bases. Consequently, hydrogen peroxide formation should make only a minor contribution to the release of unaltered bases in our hydrated DNA studies.

The G values we calculated agree well with several predictions regarding the influence of conformation and the presence of oxygen on the yield of released bases from irradiated hydrated DNA. For example, the observation that G(C) in samples irradiated under O_2 is larger than G(C) for samples irradiated under N_2 (Table IV) may be due to the scavenging of electrons by oxygen from the DNA and from the inner water of hydration (*65*), thereby reducing recombination events and increasing the number of cation radicals. A concomitant rise in base release would result, as we observed (Figs. 5, 6, 8). The fact that G(B) in oxygenated samples is smaller than G(C) in oxygenated samples implies that as Γ increases, the accessibility of O_2 to the DNA molecule appears to be impaired. This may be due to a difference in the interaction between O_2 and the pseudo-C form relative to the B form. It seems reasonable to suggest that the increased base stacking in the B form would limit the accessibility of the O_2 to the DNA bases. On the other hand, it is likely that there are differences between the reactivities of DNA base and sugar radicals and their corresponding peroxyl radicals (*27, 39, 53, 59, 60*) which will likely affect the release of unaltered bases. For example, it has been shown for DNA (*66–68*) and for adenine mononucleotides (*69*) and dinucleotides (*70*) irradiated in dilute aqueous solution that the predominance of the 3′-phosphate ester cleavage is significantly reduced in the presence of oxygen.

The results for the release of unaltered bases from DNA irradiated under O_2 are similar to the formation of other DNA lesions in solid-state DNA[1] (*71–74*). The yield of radiation-induced single- and double-strand breaks (DSBs) is enhanced by the presence of O_2 for "dry" DNA[1] (*71–74*). Evidence also indicates that radiation-induced DSBs increase and DNA–DNA crosslinks decrease as Γ is increased in the presence of O_2 (*26, 28, 74*). Any differences in the results for the formation of DSBs and crosslinks compared to the results for base release reported here probably reflect differences in the O_2 dependencies for the formation of these lesions in hydrated DNA.

Analysis of the release of the individual bases demonstrated some interesting anomalies. Overall, the amount of guanine released was approximately 60% of the other three bases (Tables I and II, Fig. 7). At $\Gamma > 15$, the rate at which each base was released as a function of Γ was not the same for adenine, cytosine, and thymine (Fig. 7). Because release of unaltered bases is considered to be derived predominantly from sugar damage that shows no sequence specificity, no difference in the yields of each released base was expected (*35*). When irradiated in dilute solution, differences in the release of the individual bases have been observed (*27, 35, 38, 75, 76*). but recent work has demonstrated that these differences disappear after the DNA is heated to 37°C for 24 h (*35*). However, even if the data shown in Fig. 7 are corrected for the additional base release shown in Table III, it is clear that the yield of the individual released bases, especially guanine, remains significantly different.

For $\Gamma < 15$, reactions involving base radicals, rather than hydroxyl radicals, are expected to contribute significantly to the yield of the released bases. The lower yield for the release of guanine at $\Gamma < 15$ may be related to the component of the DNA to which the positive hole is transferred. For the inner waters, the transfer of the positive charge from H_2O^+ to the DNA (*5, 42*) may occur at either the sugar/phosphate group (*19*) or the bases (*5, 25*). If the positive charge is localized close to a guanine or on a sugar containing a guanine residue, the charge may preferentially transfer to the guanine due to the low ionization potential of the guanine base (*77, 78*). The lower yield of guanine, compared to the other three bases, may result from the guanine radical having a lower efficiency for the production of base release than the sugar radical (*43–45*). For $\Gamma > 15$, the base

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release is due to the sum of the hydroxyl radical attack on the DNA and DNA radicals formed from charge transfer. Differences in the degree to which the individual bases were released as the number of water molecules exceeds 15 (Fig. 7) are likely to be due to differences in the reaction rates of the hydroxyl radical with the DNA bases and the sugar/phosphate group (*79*, *80*), and the efficiencies with which the subsequent base radicals form lesions that result in the release of a base (*43*).

Although our model described by Eqs. (1) and (2) provides a good working hypothesis for the treatment of base release as a function of hydration, there are other factors that have not been taken into account. For example, as Γ is varied, potential changes in prototropic effects (*81*) and/or alterations in the pH near the surface of the DNA could occur (*82*, *83*). The different levels of DNA hydration may result in changes in the degree to which various base and sugar radicals can react with water. This may prevent these radicals from undergoing protonation/deprotonation or hydration/dehydration reactions that are required for the formation of DNA strand breaks or the release of unaltered bases (*84*, *85*). For example, it has been shown for purine base radicals that protonation of these radicals can have profound effects on both their redox and their acid/base properties (*81*). Indeed, the rate for the formation of single-strand breaks by DNA base radicals is influenced by reactions of these radicals with water (*43*). This may be an important consideration in our work with hydrated DNA, because the water molecules bind predominantly to the sugar/phosphate group at low levels of hydration and then to the bases at higher levels of hydration (*14*). Additionally, there is evidence that irradiation of DNA affects the interaction between the DNA and the water of hydration, i.e., proton transfer is enhanced (*15*, *86–88*). How these factors affect the formation of DNA lesions produced from both the direct ionization of DNA and the radicals formed in the surrounding water molecules will require further study.

In summary, the release of unaltered bases from irradiated DNA depends on the level of hydration (i.e., the number of water molecules per nucleotide) and the presence or absence of O_2. The yield of released bases from the direct ionization of DNA depends on the conformation of the molecule; the *G* value for base release from the pseudo-C form of DNA (a mixture of the A and C conformers) is higher than the *G* value for the B conformer. In the presence of O_2, the *G* value for the pseudo-C form is greater by a factor of ≈ 1.7 than that in the presence of N_2 (Table IV). The efficiency for the release of individual bases from irradiated hydrated DNA differ; less guanine is released than the other three bases. Radiation damage in the first 12–15 water molecules is approximately 3.3 times less efficient in releasing unaltered bases than radiation damage in the outer, more loosely bound water molecules ($\Gamma > 15$). This ratio is similar to the relative efficiency with which hydroxyl radicals and base cations induce DNA strand breaks (*43–45*). Our results are consistent with the hypothesis that the *G* value for the first 12–15 water molecules of the DNA hydration layer is the same as the *G* value for the form of DNA to which it is bound (i.e., the pseudo-C or the B form). Thus we suggest that the release of bases originating from irradiation of the hydration waters is obtained predominantly: (1) by charge transfer from the direct ionization of the first 12–15 water molecules of the primary hydration layer and (2) by the attack of hydroxyl radicals generated in the outer, more loosely bound water molecules.

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