

Quantitation of 8-oxoG and Strand Breaks  
in pUC19 Plasmid DNA  
After Exposure to Oxygen Radical Generating Systems

by

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## ABSTRACT

Reactive oxygen species which induce a wide variety of DNA damage are produced endogenously as well as through exposure to environmental chemicals and ionizing radiation. Often, a specific DNA lesion is used as an index of the entire spectrum of oxidative DNA damage products for an oxygen radical generating system. To investigate the correlation between a single product and total damage, we measured both strand breaks and 8-oxoG in DNA after exposure to gamma radiation, Fe(II)-EDTA and H<sub>2</sub>O<sub>2</sub>, Cu(II) and H<sub>2</sub>O<sub>2</sub>, and peroxynitrite at concentrations of physiological relevance. We found the ratio of 8-oxoG to strand breaks varied more than 10-fold depending on the oxidizing agent, with Cu(II)/H<sub>2</sub>O<sub>2</sub> and peroxynitrite producing approximately the same higher ratios and Fe(II)-EDTA/H<sub>2</sub>O<sub>2</sub> and gamma radiation producing similar lower ratios. In addition to the variation between agents, the relative proportion of 8-oxoG and strand breaks varied more than 2-fold for different concentrations of Cu(II)/H<sub>2</sub>O<sub>2</sub>, demonstrating the ratio to be a function of concentration for a single oxidizing agent. These results indicate that a single oxidative DNA product is not representative of the total DNA damage in comparisons of different oxidizing systems or for different concentrations of a single agent. Thus, the chemistry of the oxygen radical generating systems must be considered when studying oxidative DNA damage as different oxidizing agents may produce different spectrums of damage depending on the intermediates involved.

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## ABBREVIATIONS

8-oxoguanine	8-oxoG
N <sup>2</sup> -methyl-8-oxoguanine	N <sup>2</sup> -methyl-8-oxoG
Copper(I)	Cu(I)
Copper(II)	Cu(II)
Deoxyribonucleic acid	DNA
Diethylenetriaminepentaacetic acid	DETAPAC
Ethylenediaminetetraacetic acid	EDTA
High pressure liquid chromatography coupled with electrochemical detection	HPLC HPLC-ECD
Hydrogen peroxide	H <sub>2</sub> O <sub>2</sub>
Hydroxyl radical	OH <sup>·</sup>
Iron(II)	Fe(II)
Monoclonal antibodies	MAb
Peroxynitrite	ONOO <sup>-</sup>

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# INTRODUCTION

## Oxidative Stress

Oxidative DNA damage resulting from exposure to reactive oxygen species has been proposed to have a role in a variety of biological processes such as mutagenesis, aging and carcinogenesis (1). Reactive oxygen species are generated endogenously through normal cellular metabolism, inflammation, ischemia and xenobiotic metabolism (2). In addition, many environmental chemicals and ionizing radiation also produce oxygen radicals (3). Transition metals, such as iron and copper, can also catalyze the formation of reactive oxygen species from hydrogen peroxide via a Fenton-like reaction (4).

Reactive oxygen species induce a wide variety of DNA damage, including single and double strand breaks, modified bases, abasic sites, and DNA-protein crosslinks (5). Therefore, free radicals may be both mutagenic and carcinogenic (6). Neither superoxide ( $O_2^{\cdot-}$ ) nor hydrogen peroxide ( $H_2O_2$ ) alone cause strand breaks or chemical modifications of DNA at physiological concentrations, however, hydroxyl radicals ( $OH^{\cdot}$ ) and singlet oxygen ( $^1O_2$ ) can directly attack DNA leading to oxidative damage (7). Antioxidant defenses, which include enzymes and low molecular mass free radical scavengers, have evolved for protection from reactive oxygen species, and the generation of reactive oxygen species is usually balanced with antioxidant defenses (8). Oxidative stress results when antioxidants are depleted and/or the formation of reactive oxygen species is increased (8).

## **Biomarkers of Oxidative Damage**

DNA single strand breaks result from oxidative damage and are often used as a measurement of oxidative stress (9). If unrepaired, single strand breaks can form double strand breaks which appear to be the most lethal DNA lesion produced by free radicals (10). Strand breaks in DNA can result in cell death, cessation of proliferative capacity, mutations and/or malignant transformations (11). Oxygen radicals can attack DNA at a sugar which will ultimately give rise to sugar fragmentation, base loss and a strand break with a terminal fragmented sugar residue (12). The initial reaction leading to strand breaks is the abstraction of a hydrogen atom from the deoxyribose of DNA which requires a strong oxidant (13) (Figure 1). Abasic sites are also formed after radical attack on the deoxyribose and can react to ultimately generate strand breaks.

The oxidative DNA damage product 8-oxoguanine (8-oxoG) was discovered in 1983 by Kasai and Nishimura (14) (Figure 2). In 1986, Floyd et al. reported the development of a sensitive analytical technique for 8-oxoG in DNA involving HPLC coupled with electrochemical detection (15) making 8-oxoG a useful marker for monitoring oxidative DNA damage in studies of various oxygen radical forming agents (3). The role of 8-oxoG in mutagenesis and carcinogenesis has been widely investigated (16) and many studies have shown a correlation between the formation of 8-oxoG in DNA and carcinogenesis (17). 8-oxoG is predicted to assume a syn conformation in DNA (17) (Figure 3) and has been found to give rise predominantly to G → T transversions (3, 18).

Hydroxyl radicals have been demonstrated to mediate the formation of 8-oxoG in

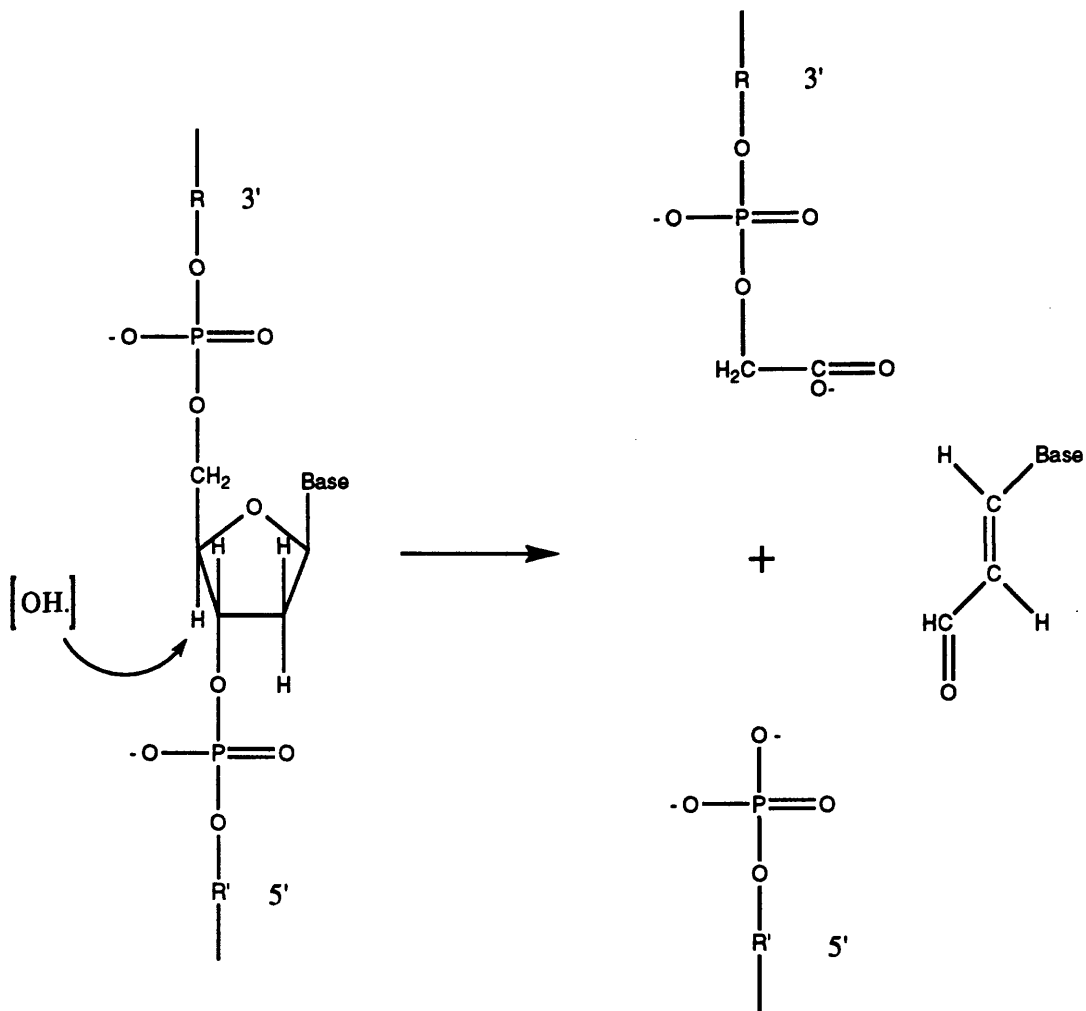
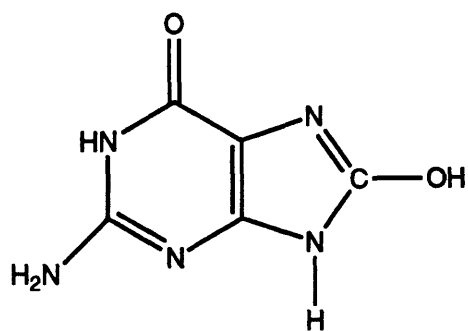
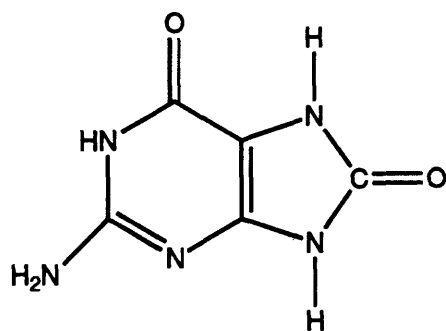


Figure 1. A possible mechanism for the formation of single strand breaks by hydroxyl radical attack on the deoxyribose (12).

a.



b.



c.

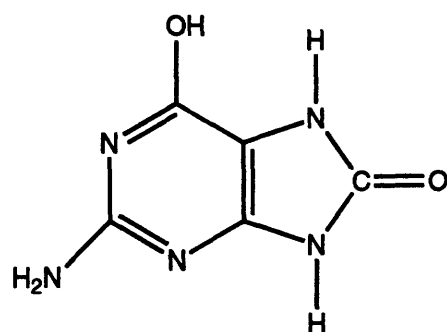
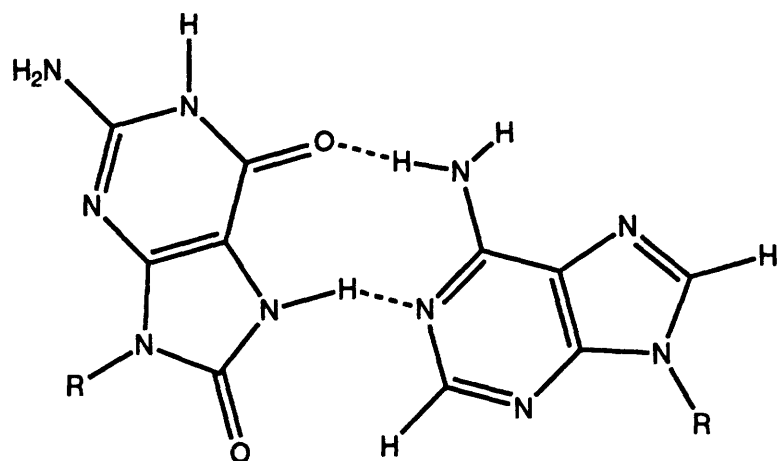


Figure 2. The chemical structure of 8-oxoG: a. 6-Keto, 8-Enol form. b. 6,8-DiKeto form. c. 6-Enol, 8-Keto form (17).

a.



b.

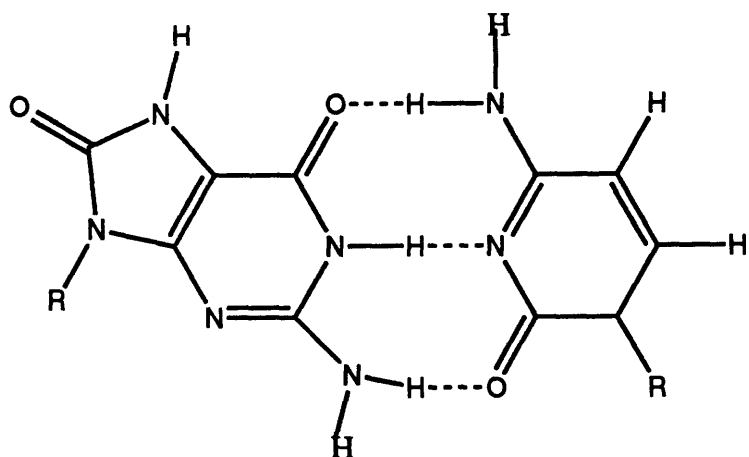
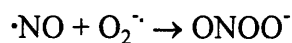


Figure 3. 8-oxoG base pairs: a. 8-oxoG(*syn*):dA(*anti*). b. 8-oxoG(*anti*):dC(*anti*) (18).

DNA (19). This reaction is thought to involve the addition of OH· to the C-8 of guanine, followed by the subsequent loss of hydrogen atom, or the one-electron oxidation of the guanine by an oxidizing agent and subsequent addition of water (17) (Figure 4). Singlet oxygen (16) and specific oxidants (17) have also been found to form 8-oxoG. Thus, oxygen radicals can give rise to both strand breaks and 8-oxoG depending on whether attack occurs on the deoxyribose or at the base.

### **Peroxynitrite**

Nitric oxide ( $\cdot\text{NO}$ ) is an endogenously formed molecule involved in the mediation of many biological processes (20). Nitric oxide can react with superoxide to form peroxynitrite ( $\text{ONOO}^-$ ) with a rate constant near the diffusion-controlled limit in the following reaction (21):



Nitric oxide and superoxide are produced simultaneously by many cell types, including Kupffer cells, neutrophils, endothelial cells and macrophages (22). At these loci, the concentrations of both nitric oxide and superoxide would be significant and favor the formation of peroxynitrite in vivo (23).

Peroxynitrite and its conjugate acid, peroxynitrous acid ( $\text{HOONO}$ ), are potent oxidants which are capable of oxidizing a variety of biomolecules, including thiols, deoxyribose and lipids (23). At physiological pH, peroxynitrite is highly reactive through at least three different oxidative pathways which include a hydroxyl radical-like intermediate, a direct reaction with sulfhydryl groups and a reaction with metal ions

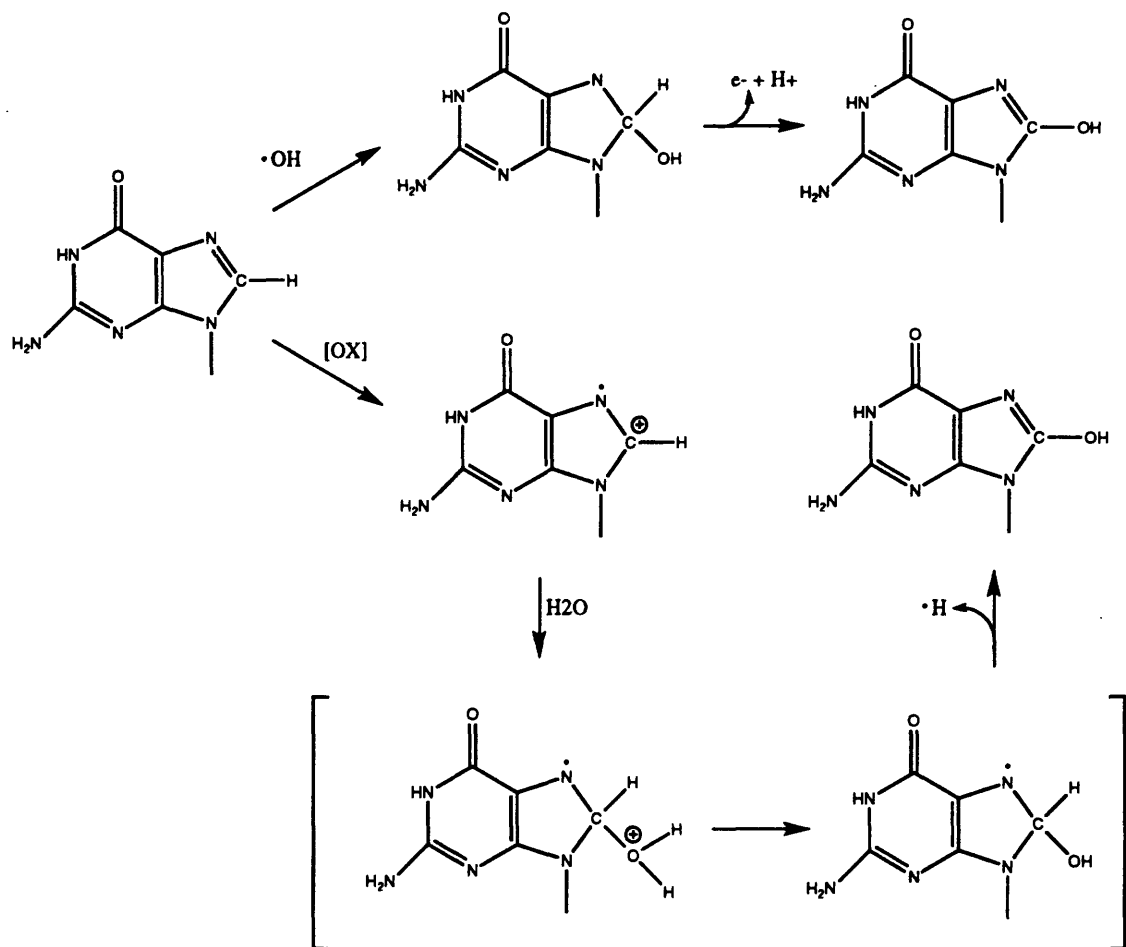


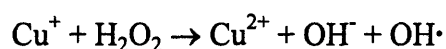
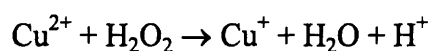
Figure 4. Possible mechanisms for the formation of 8-oxoG by addition of  $\text{OH}\cdot$  to the C-8 of guanine or the one-electron oxidation of the C-8 position by an oxidizing agent and subsequent addition of water (17).

to form a nitrating species (24). A high-energy form of peroxyxynitrite, HOONO\*, has been proposed to be the oxidizing species which reacts like the hydroxyl radical but with greater specificity (23). Oxidations by peroxyxynitrite can occur through either one- or two-electron reactions (25).

The majority of peroxyxynitrite-induced mutations have been found at G:C base pairs and predominantly involve G → T transversions (26). Peroxyxynitrite has been proposed to be the oxidizing agent responsible for the formation of 8-oxoG in activated macrophages (27). Recently, peroxyxynitrite has also been demonstrated to cause single strand breaks in plasmid DNA (28).

## **Copper**

Copper is an essential element and has been reported to play a significant role in maintenance of nuclear matrix organization and DNA folding (29). The N-7 position of guanine has been found to be susceptible to covalent binding by copper(II) (30, 31). Copper(II) in the presence of excess hydrogen peroxide has been proposed to react to generate hydroxyl radicals through the following reactions (32, 33):



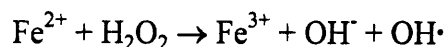
If these reactions occur with copper bound to DNA, then the OH<sup>•</sup> immediately attacks the DNA in a site-specific reaction (7).

Sequencing experiments have shown that sequences near guanine residues are preferentially damaged by copper(II) and hydrogen peroxide (1, 34). The most frequent

mutations induced by copper are C → T transitions, followed by G → T transversions (35). Copper(II) in the presence of hydrogen peroxide has been demonstrated to damage DNA through the formation of both single strand breaks (1, 5, 34, 36) and base modifications, including 8-oxoG (7, 37).

## **Iron**

Iron is essential for oxygen transport and the function of many enzymes. Most iron is complexed in nature, but a variety of xenobiotics has been found to facilitate the release of iron (4). Free iron can act as a catalyst in hydroxyl radical formation from hydrogen peroxide in the Fenton reaction (38):



The EDTA complex of iron(II) when reacted with hydrogen peroxide has been shown to generate the hydroxyl radical (39). Due to the negative charge of the complex, it does not electrostatically associate with DNA, and unlike Cu(II), Fe(II)-EDTA is not believed to bind DNA (39, 40). As a result, the OH· which is formed exists as a free radical in the fluid phase and has been shown to generate strand breaks with virtually no base sequence specificity (39, 40). The oxygen radicals produced in the reaction of iron(II) with hydrogen peroxide have been shown to be mutagenic (41).

## **Gamma radiation**

Ionizing radiation also produces OH· and other radical species, such as hydrated electron and H atom, through the homolytic fission of oxygen-hydrogen bonds in water (6). The hydrated electron can react with N<sub>2</sub>O, when present in solution, to generate additional OH· (6). As with Fe(II)-EDTA, the radical species which are formed are free

in solution (34). Radiation-generated  $\text{OH}\cdot$  can react with both the bases and the deoxyribose in DNA and has been shown to form both strand breaks (13, 34) and 8-oxoG (42). In addition, exposure to background levels of ionizing radiation has been proposed to be a source of hydroxyl radicals in all living organisms (9).

### **Aims of This Research**

In many experiments, a specific DNA lesion is used as a representative measurement of the entire spectrum of oxidative DNA damage products for a specific oxygen radical generating system. We decided to investigate the premise that a single product can represent all oxidative damage through the measurement of both single strand breaks and 8-oxoG after exposure of plasmid DNA to peroxyxynitrite, copper(II) and hydrogen peroxide, iron(II)-EDTA and hydrogen peroxide, and gamma radiation at different concentrations approaching physiological relevance. Single strand breaks and 8-oxoG were used to compare the reactivity of the deoxyribose versus the base in DNA to reactive oxygen species.

We found that the ratio of 8-oxoG to strand breaks was not constant for the different oxygen radical generating systems. In addition, the relevant proportions of 8-oxoG and strand breaks were found to vary as a function of the concentration for certain oxidizing agents. Our results indicate that different oxygen radical generating systems do not necessarily react identically with DNA. This dissimilarity in chemistry may arise from different reactive oxygen species and/or through a difference in the specificity of binding of the agent to DNA. These results bring into question the use of a single

biomarker as an indicator of oxidative damage, either for different oxidizing agents or different concentrations of a single agent.

## MATERIALS AND METHODS

**Chemicals.** 2-amino-6-8-dihydroxypurine (8-oxoG) was obtained from Chemical Dynamics Corp. pUC19 plasmid DNA was obtained from New England Biolabs. Cyanogen bromide-activated sepharose 4B, N<sup>2</sup>-methyl guanosine, diethylenetriaminepentaacetic acid (DETAPAC) and PBS were purchased from Sigma. Cupric chloride, ethylenediaminetetraacetic acid (EDTA), potassium phosphate (mono- and dibasic) and acetonitrile were obtained from Mallinckrodt. Hydrogen peroxide (30%) and ammonium acetate were purchased from Fisher. Methylsulfoxide (DMSO) and 98% formic acid were obtained from EM Science. Chelex 100 Resin and poly-prep columns were purchased from BioRad Laboratories. SpectraPor 7 Membranes with a MWCO of 1000 were obtained from Spectrum. Bromine was purchased from Aldrich. [<sup>3</sup>H]8-oxoG was generously provided by Dr. William Boadi. Plasmid Giga purification kit was purchased from Qiagen.

**HPLC System.** The HPLC-ECD system consisted of a Hewlett Packard model 1050 pump, an ESA model 5100A coulchem electrochemical detector and an ESA model 5010 analytical cell. Separation of nucleobases was achieved using a Supelco LC-18-DB 5 μm column (25 cm x 4.6 mm) under isocratic conditions. The mobile phase was 2% methanol in 50 mM ammonium acetate, pH 5.5 with a flow rate of 1 mL/min.

**Synthesis of N<sup>2</sup>-methyl-8-oxoG.** ~ 500 μg of N<sup>2</sup>-methyl guanosine was dissolved in 500 μL of double distilled water. 40 μL of brominated water (1:200 Br<sub>2</sub> in H<sub>2</sub>O) was added and inverted to mix. N<sup>2</sup>-methyl-8-bromo-guanosine was purified from

unreacted N<sup>2</sup>-methyl guanosine by HPLC using an Alltech C18 column (25 cm x 4.6 mm) with 50 mM ammonium acetate, pH 5.5 as mobile phase and monitoring at 260 nm. N<sup>2</sup>-methyl-8-bromo-guanosine eluted at ~ 14 min, was collected manually and then dried by centrifugation under vacuum (0.05 mbar). 500 µL of formic acid was added, reacted for 16 hours at 130°C and then dried by centrifugation under vacuum. The reaction with formic acid was repeated and dried again by centrifugation under vacuum. The product was redissolved in 50 mM ammonium acetate, pH 5.5, filtered and purified by HPLC using an Alltech C18 column (25 cm x 4.6 mm) with 2% methanol in water as mobile phase and monitoring at 260 nm. The fraction which eluted at 14 min was collected and dried by centrifugation under vacuum. The fraction was then derivatized by adding 15 µL acetonitrile, 10 µL pyridine and 25 µL BSTFA and reacting at 130°C for 20 minutes. The product was identified as N<sup>2</sup>-methyl-8-oxoguanine by GC/MS. The product was dried by centrifugation under vacuum and stored at -20°C.

**Standard Solutions.** Commercial 8-oxoG and purified N<sup>2</sup>-methyl-8-oxoG were dissolved in double distilled water and the concentration was determined by UV at pH 9.5 using an extinction coefficient of  $8.14 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$  at 283 nm (43) using a Hewlett Packard model 8459A UV/VIS Spectrophotometer. The solutions were adjusted to 10 pg/µL. Aliquots of 1.5 mL of the N<sup>2</sup>-methyl-8-oxoG solution were stored at -80°C. The 8-oxoG solution was stored at -20°C and a fresh solution was prepared every several months.

**Preparation of buffer.** 2 liters of 50 mM potassium phosphate, pH 7.4 were prepared from the mono- and dibasic salts in double distilled water. Chelex 100 resin

was added to the buffer (2.5 g resin/L solution) and stirred at room temperature for at least 2 hours to remove any trace metals. The buffer was filtered on a Buchner funnel with # 576 analytical filter paper (Schleicher & Schuell) and Chelex resin was added twice more, being stirred for > 2 hours and filtered each time. The buffer was stored at 4°C and aliquots were removed when needed. Fresh aliquots were used for each treatment.

**Synthesis of Peroxynitrite.** Peroxynitrite solution was prepared as described by Pryor, et al. (44). Briefly, ozone, generated through a Welsbach ozonator, was bubbled into 100 mL of 0.1 M sodium azide in water, chilled in an ice bath. After 45 minutes of ozonation, the peroxynitrite was estimated spectrophotometrically after a 100-fold dilution in 0.1 N NaOH ( $\epsilon = 1670 \text{ M}^{-1}\text{cm}^{-1}$  for peroxynitrite at 302 nm (44)) and stored at -80°C. The reactivity of the peroxynitrite was analyzed through the nitration of L-tyrosine using the method described by Beckman, et al. (45) and modifications by Pryor, et al. (44).

**Preparation and purification of pUC19 plasmid DNA.** LB plates were prepared by pouring 35 mL of autoclaved broth (5 g tryptone, 2.5 g yeast extract, 2.5 g NaCl, 7.5 g Agar and 0.5 mL 1 N NaOH in 500 mL double distilled water with 40  $\mu\text{L}$  of ampicillin (10 mg/mL)) onto each plate and allowing to solidify. In preparation for transforming cells, pUC19 stock (1  $\mu\text{g}/\mu\text{L}$ ) was diluted 5-fold in double distilled water and 1  $\mu\text{L}$  was then added to 100  $\mu\text{L}$  of DH5a *E. coli* cells and incubated on ice for 45 minutes. 800  $\mu\text{L}$  sterile LB medium (12 g tryptone, 6 g yeast extract, 6 g NaCl and 1.2 mL 1 N NaOH in 1.2 L double distilled water) and 100  $\mu\text{L}$  200 mM glucose were added

and incubated with constant shaking at 37°C for 2 hours. 50 and 100 µL aliquots of the transformed cells were plated on LB plates and incubated for >15 hours. Colonies from the agar plates were then added to a feeder culture (10 mL of LB medium and 100 µg/mL sterile filtered ampicillin) and incubated at 37°C with constant shaking overnight. The feeder was then added to 1 L culture and incubated 12 hours.

Cells were spun down at 15,000 x g for 15 minutes and supernatant was removed from pellet. Plasmid DNA was isolated using the Qiagen Giga Plasmid/Cosmid Purification Protocol. After washing DNA pellet with 80% ethanol, the ethanol was removed and the DNA was redissolved in 50 mM potassium phosphate, pH 7.4, treated with Chelex. Plasmid DNA was dialyzed against 50 mM potassium phosphate containing 1 mM DETAPAC for 12 hours at 4°C to remove trace metals and then against 50 mM potassium phosphate for an additional 12 hours at 4°C.

Plasmid DNA was removed from dialysis and the concentration was determined using the Fluorescent Hoechst Dye. Briefly, 5 µg of standard pUC19 of known concentration and isolated DNA were HindIII digested at 37°C for 1 hour and 15 minutes to linearize the DNA. The pUC19 standard DNA were then aliquoted to give a standard curve and the volume of the standard and isolated DNA was brought to 2 mL in buffer H (10 mM Tris, 1 mM EDTA and 100 mM NaCl, pH 7.0). 1 mL of Hoescht dye (1 mg dye in 12.5 mL of buffer H, diluted 100-fold in buffer H) was added and incubated at room temperature for 2 minutes in dim light. The fluorescence was measured using a model 450 fluorometer, the standard curve was plotted and the concentration of the isolated

DNA was determined from the equation for the standard curve. The pUC19 DNA was stored in 200  $\mu\text{g}$  aliquots at  $-80^{\circ}\text{C}$ .

**Coupling of monoclonal antibodies to CNBr-activated Sepharose 4B.** The monoclonal antibodies were produced as described previously by Ravanat, et al. (46). Ascites serum was purified by ammonium sulfate precipitation (40% w/v), dialyzed against coupling buffer (0.1 M  $\text{NaHCO}_3$  and 0.5 M  $\text{NaCl}$ , pH 8.3) and bound to CNBr-activated Sepharose 4B (2 mg protein/mL gel), which had been swelled in 1 mM  $\text{HCl}$ , as described previously (47, 48). The gel was stored at  $4^{\circ}\text{C}$  in phosphate buffered saline with 0.02% sodium azide. Prior to use in immunoaffinity columns, the antibodies were washed with 50% DMSO (25 x gel volume) on a scintered glass filter to remove existing bound 8-oxoG. The antibodies were then washed with an equal volume of water and collected in PBS. The binding efficiency of the antibodies was determined to be 75 to 80% through the use of  $[^3\text{H}]8\text{-oxoG}$ .

**Treatment of pUC19 plasmid with Cu(II).** The cupric chloride, EDTA and hydrogen peroxide solutions were prepared fresh for every treatment.  $\sim 17.28$  mg of  $\text{CuCl}_2$  was dissolved in 1 mL of d.d. water, diluted 100-fold to obtain a 1 mM solution, and then 50, 75, 100, 125, 150, 200, 250 or 300  $\mu\text{L}$  was brought to 1 mL for final concentrations of 50, 75, 100, 125, 150, 200, 250 or 300  $\mu\text{M}$  Cu(II). 10  $\mu\text{L}$  of hydrogen peroxide (6.2 M solution as determined by UV for a 1:500 dilution in water at 230 nm with  $\epsilon = 81 \text{ M}^{-1}\text{cm}^{-1}$ ) was added to 610  $\mu\text{L}$  of 50 mM potassium phosphate, pH 7.4, treated with Chelex and then diluted 10-fold for a final concentration of 10 mM.  $\sim 37.22$  mg of EDTA was dissolved in 1 mL of 50 mM potassium phosphate, pH 7.4, treated with

Chelex, diluted 10-fold to obtain a 10 mM solution and then 50, 75, 100, 125, 150, 200, 250 or 300  $\mu\text{L}$  was brought to 1 mL for final concentrations of 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5 or 3 mM EDTA.

200  $\mu\text{g}$  plasmid DNA was added to 50 mM potassium phosphate, pH 7.4, treated with Chelex. 0.67 mL of Cu(II) and 0.67 mL of hydrogen peroxide was added to the DNA solution for a final volume of 6.67 mL. The solution was mixed immediately and reacted for 30 minutes at room temperature. The final reaction contained 30  $\mu\text{g}$  DNA/mL with 5, 7.5, 10, 12.5, 15, 20, 25 or 30  $\mu\text{M}$  Cu(II) and 1 mM  $\text{H}_2\text{O}_2$ . After 30 minutes, 10-fold excess EDTA was added and the solution was moved to 4°C.

**Treatment of pUC19 plasmid with Peroxynitrite.** A fresh aliquot of peroxynitrite was used for every treatment.  $\text{ONOO}^-$  was removed from -80°C and diluted 100-fold in 0.1 N NaOH. The concentration was determined by UV at 302 nm using an extinction coefficient of  $1670 \text{ M}^{-1} \text{ cm}^{-1}$  (44).  $\text{ONOO}^-$  was then diluted in 0.1 N NaOH to obtain a 10 mM solution and 0.25, 0.5, 1, 2.5, 5, 10, 15, 20, 25 or 50  $\mu\text{L}$  was brought to 1 mL for final concentrations of 2.5, 5, 10, 25, 50, 100, 150, 200, 250 or 500  $\mu\text{M}$   $\text{ONOO}^-$ .

200  $\mu\text{g}$  plasmid DNA was added to 50 mM potassium phosphate, pH 7.4, treated with Chelex resin. 0.67 mL of  $\text{ONOO}^-$  was added to the DNA solution for a final volume of 6.67 mL, mixed immediately and reacted at room temperature for 30 minutes. The final reaction contained 30  $\mu\text{g}$  DNA/mL and 0.25, 0.5, 1, 2.5, 5, 10, 15, 20, 25 or 50  $\mu\text{M}$   $\text{ONOO}^-$ . After 30 minutes, the solution was moved to 4°C.

**Treatment of pUC19 plasmid with Fe(II)/EDTA.** The ferrous ammonium sulfate, EDTA and hydrogen peroxide solutions were prepared fresh for every treatment.

~39.22 mg of  $\text{FeNH}_4\text{SO}_4 \cdot 6\text{H}_2\text{O}$  was dissolved in 1 mL of d.d. water, diluted 100-fold to obtain a 1 mM solution, and then 5, 10, 15, 20, 25, 50 or 100  $\mu\text{L}$  was brought to 1 mL for final concentrations of 5, 10, 15, 20, 25, 50 or 100  $\mu\text{M}$  Fe(II). 10  $\mu\text{L}$  of hydrogen peroxide was added to 610  $\mu\text{L}$  of 50 mM potassium phosphate, pH 7.4, treated with Chelex and then diluted 10-fold for a final concentration of 10 mM. ~37.22 mg of EDTA was dissolved in 1 mL of 50 mM potassium phosphate, pH 7.4, treated with Chelex, diluted 100-fold to obtain a 1 mM solution and then 10, 20, 30, 40, 50, 100 or 200  $\mu\text{L}$  was brought to 1 mL for final concentrations of 10, 20, 30, 40, 50, 100 or 200  $\mu\text{M}$  EDTA.

200  $\mu\text{g}$  plasmid DNA was added to 50 mM potassium phosphate, pH 7.4, treated with Chelex. 0.67 mL of hydrogen peroxide, 0.67 mL of EDTA and 0.67 mL of Fe(II) was added to the DNA solution for a final volume of 6.67 mL. The solution was mixed immediately and reacted for 30 minutes at room temperature. The final reaction contained 30  $\mu\text{g}$  DNA/mL with 0.5, 1, 1.5, 2, 2.5, 5 or 10  $\mu\text{M}$  Fe(II), 1, 2, 3, 4, 5, 10 or 20  $\mu\text{M}$  EDTA and 1 mM  $\text{H}_2\text{O}_2$ . After 30 minutes, the solution was moved to 4°C.

**Treatment of pUC19 plasmid with gamma radiation.** Solutions of plasmid DNA (30  $\mu\text{g}/\text{mL}$ ) were prepared in 50 mM potassium phosphate, pH 7.4, treated with Chelex. Solutions were saturated with air or  $\text{N}_2\text{O}$ . DNA was irradiated using a  $^{60}\text{Co}$   $\gamma$  source with a dose rate of 4 Gy/min to give the 0, 0.125, 0.375, 0.5, 1, 5 and 10 Gy exposures. DNA was incubated on ice for 30 minutes following irradiation.

**Preparation of pUC19 random primer probe.** 1.75  $\mu\text{L}$  of 50 ng/ $\mu\text{L}$  DdeI digested pUC19 DNA, 1.25  $\mu\text{L}$  of random hexamer ( $\text{dN}_6$ ), 10  $\mu\text{L}$  of buffer, 1  $\mu\text{L}$  100

$\mu\text{g}/\mu\text{L}$  BSA, 10  $\mu\text{L}$  of [ $^{32}\text{P}$ ] $\alpha\text{dATP}$  (10  $\mu\text{Ci}/\mu\text{L}$ ) and 1  $\mu\text{L}$  of T4 polymerase (Klenow fragment 1  $\mu\text{g}/\mu\text{L}$ ) were incubated at 37°C for 4 hours and then placed in a 65°C waterbath for 3 min to denature the polymerase. 1  $\mu\text{L}$  of 500  $\mu\text{M}$  EDTA was added and volume was brought to 50  $\mu\text{L}$  with double distilled water. DNA was phenol/chloroform extracted twice and then chloroform/isoamyl alcohol extracted twice to remove protein. Probe was spun through a G-50 sephadex column to remove unincorporated [ $^{32}\text{P}$ ]dATP and then probe was counted by scintillation.

**Quantitation of strand breaks.** After treatment, 1.5  $\mu\text{g}$  of pUC19 plasmid DNA (30  $\mu\text{g}/\text{mL}$ ) was exposed to 100 mM putrescine for 1 hour at 37°C to cleave abasic sites (10). 10  $\mu\text{L}$  of DNA was loaded on a 1% agarose 1x TBE high melt agarose gel and run at 3 volts/cm for 4 hours. The gel was dried on a conventional gel dryer for 30 minutes at room temperature and 30 minutes at 60°C. Gel was soaked in ~10 gel volumes of .25 M HCl for 30 minutes. Gel was rinsed with double distilled water and soaked in denaturing solution (.5 M NaOH and .15 M NaCl) for 20 minutes and then rinsed and soaked again. Gel was then rinsed with double distilled water and soaked in neutralizing solution (.5 M TrisHCl and .15 M NaCl, pH 7.2) for 20 minutes and then rinsed and soaked again. Gel was placed in hybridization bottle containing 5 mL of prehybridization/hybridization solution (6x SSC and 1x SPED) and 100  $\mu\text{g}/\text{mL}$  salmon testes DNA, denatured by boiling, was added. Gels were then pre-hybridized at 55°C for at least 1 hour. Solution was decanted and replaced with 5 mL fresh prehybridization/hybridization solution, 100  $\mu\text{g}/\text{mL}$  salmon testes DNA and  $10^6$  cpm/mL plasmid probe, denatured by boiling. Probe was hybridized at 55°C overnight. Solution was replaced with 5 mL of wash solution

(0.1% SSC and 0.5% SDS) and placed in hybrid oven at 55°C for 30 minutes and then repeated twice more.

The fraction of form I and form II plasmid was then determined by autoradiography using a Molecular Dynamics phosphorimager.

**DNA hydrolysis.** After treatment, pUC19 plasmid DNA was dialyzed against double distilled water for 16 to 20 hours. The concentration of the solution was determined by UV at 260 nm (1 O.D. corresponding to 50 µg) and 50 µg was aliquotted in triplicate into screw cap vials. The DNA was then dried by centrifugation under vacuum (0.05 mbar). 190 µL of H<sub>2</sub>O and 310 µL of 98% formic acid were added to give 60% formic acid and hydrolysis was performed for 1 hour at 100°C. Formic acid was then removed by centrifugation under vacuum.

**Purification on immunoaffinity columns.** 8-oxoG was purified on immunoaffinity columns as described by Ravanat, et al. (46), but with the following modifications. 0.2 mL of gel bound with antibodies was packed on Bio-Rad poly-prep columns. Hydrolyzed DNA was resuspended in 0.5 mL of PBS and applied to the column 3 times. The column was then washed under gravity induced flow with 2.0 mL of PBS, 2.0 mL of H<sub>2</sub>O and 1 mL of acetonitrile. The bound 8-oxoG was then eluted with 1.5 mL of MeOH. MeOH was cooled at -20°C for 30 minutes and 150 µL of N<sup>2</sup>-methyl-8-oxoG solution (10 pg/µL) was added. Elution was then dried by centrifugation under vacuum. The dried residue was redissolved in 50 µL of the HPLC mobile phase prior to injection onto the HPLC-ECD apparatus.

**Quantitation of 8-oxoG.** The areas under the 8-oxoG and the N<sup>2</sup>-methyl-8-oxoG peaks were determined by integration and the ratio of 8-oxoG to N<sup>2</sup>-methyl-8-oxoG was calculated. From a calibration curve relating known amounts of 8-oxoG to N<sup>2</sup>-methyl-8-oxoG, the ratio was converted to pg 8-oxoG and then the number of 8-oxoG per 10<sup>6</sup> bases was calculated.

## RESULTS

**Nitration of L-tyrosine by Peroxynitrite.** The activity of synthesized peroxynitrite was assessed through a reaction with L-tyrosine to form nitrotyrosine. Peroxynitrite (0, 0.4, 0.8, 1.2 and 1.6 mM) was reacted with L-tyrosine (0.8 mM) in 80 mM potassium phosphate, pH 7.4 for 10 minutes at room temperature. The concentration of nitrotyrosine was then determined by UV at 438 nm using an extinction coefficient of  $4200 \text{ M}^{-1}\text{cm}^{-1}$  (45). The concentration of nitrotyrosine was found to be 0, 0.018, 0.034, 0.042 and 0.062 mM for 0, 0.4, 0.8, 1.2, and 1.6 mM  $\text{ONOO}^-$ , respectively, which gives a 3.7% mole yield (Figure 5) and corresponds to values reported previously by Beckman (6.9%) (45) and Pryor (7.3%) (44).

**8-oxoG determination by HPLC-ECD.** Following elution from MAb column and addition of  $\text{N}^2$ -methyl-8-oxoG, the solution was injected onto a C-18 reversed phase column connected to the coulometric detector. 8-oxoG and  $\text{N}^2$ -methyl-8-oxoG elute at approximately 7 and 20 min, respectively (Figure 6). The calibration curve was determined using varying amounts of 8-oxoG and 1500 pg of  $\text{N}^2$ -methyl-8-oxoG. The calibration curve (Figure 7) was found to be linear from 200 pg to 2 ng of 8-oxoG injected (correlation coefficient = 0.998).

### **Peroxynitrite**

Peroxynitrite has recently been reported to form single strand breaks in DNA in vitro (28) and 8-oxoG in activated macrophages (27) and was therefore chosen to be investigated as an oxidizing agent. 30  $\mu\text{g/mL}$  pUC19 plasmid DNA was treated with

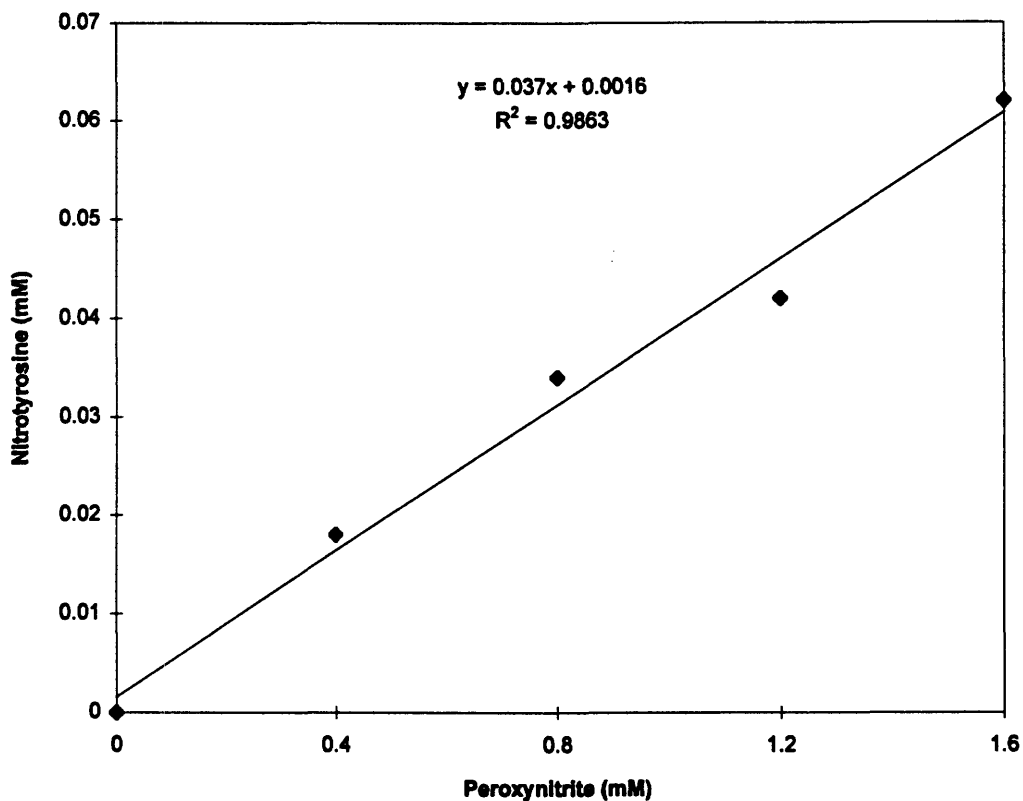


Figure 5. 0, 0.4, 0.8, 1.2 and 1.6 mM of  $\text{ONOO}^-$  were reacted with 0.8 mM L-tyrosine for 10 minutes at room temperature in 80 mM potassium phosphate, pH 7.4. Reaction was quenched by the addition of 0.25 mL of 0.1 N NaOH. The concentration of nitrotyrosine was determined at 438 nm using an extinction coefficient of  $4200 \text{ M}^{-1} \text{ cm}^{-1}$ . The mole yield was determined to be 3.7% from the slope of the peroxynitrite concentration versus the concentration of nitrotyrosine.

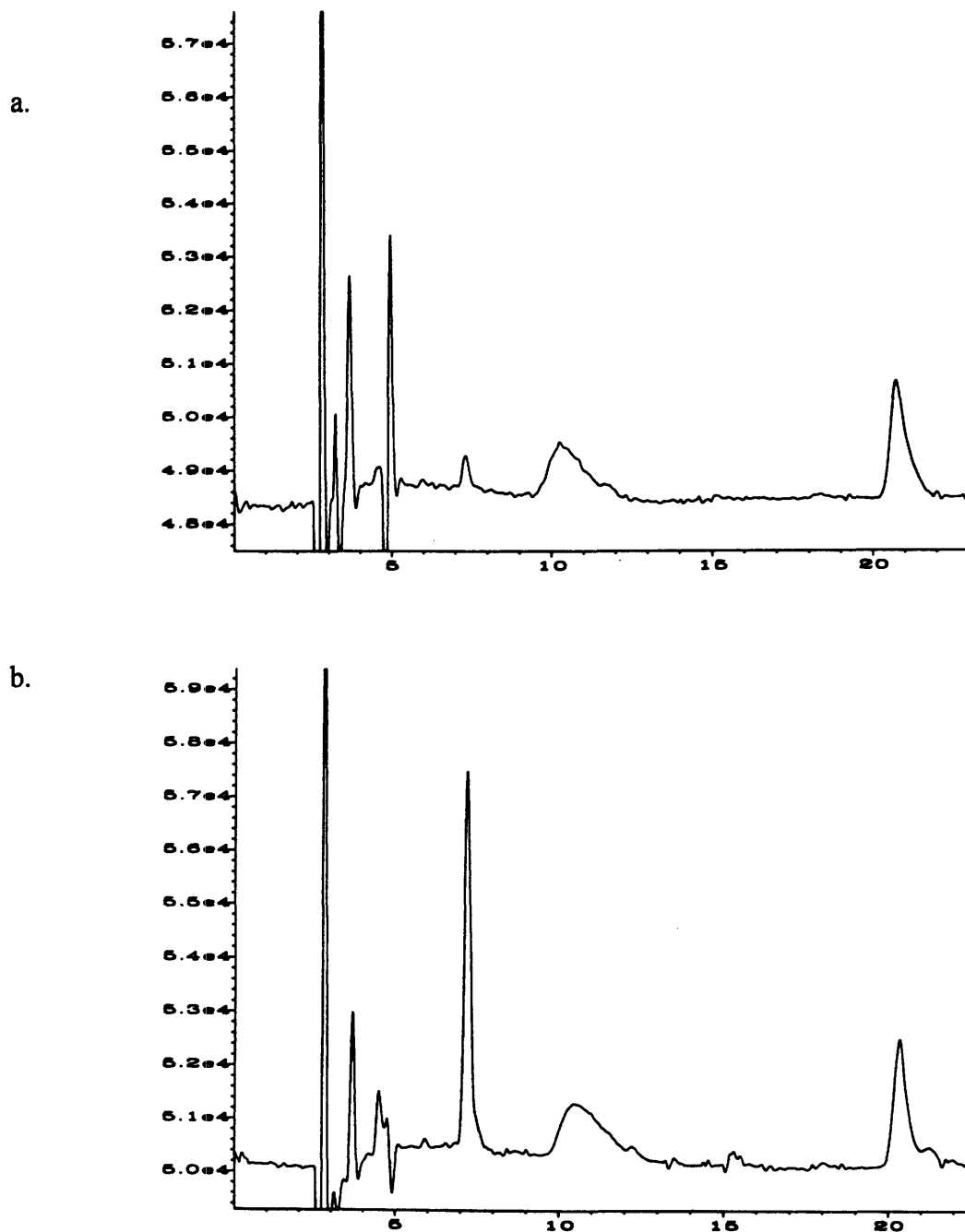


Figure 6. HPLC-ECD chromatograms of 8-oxoG and N<sup>2</sup>-methyl-8-oxoG in a: untreated pUC19 plasmid and b: plasmid treated with 30 μM Cu(II). The nucleobases were separated using a Supelco LC-18-DB 5 μm column (25 cm x 4.6 mm) and an isocratic elution with 2% methanol in 50 mM ammonium acetate, pH 5.5 as the mobile phase. 8-oxoG eluted at ~ 7 minutes and N<sup>2</sup>-methyl-8-oxoG at ~ 20 minutes.

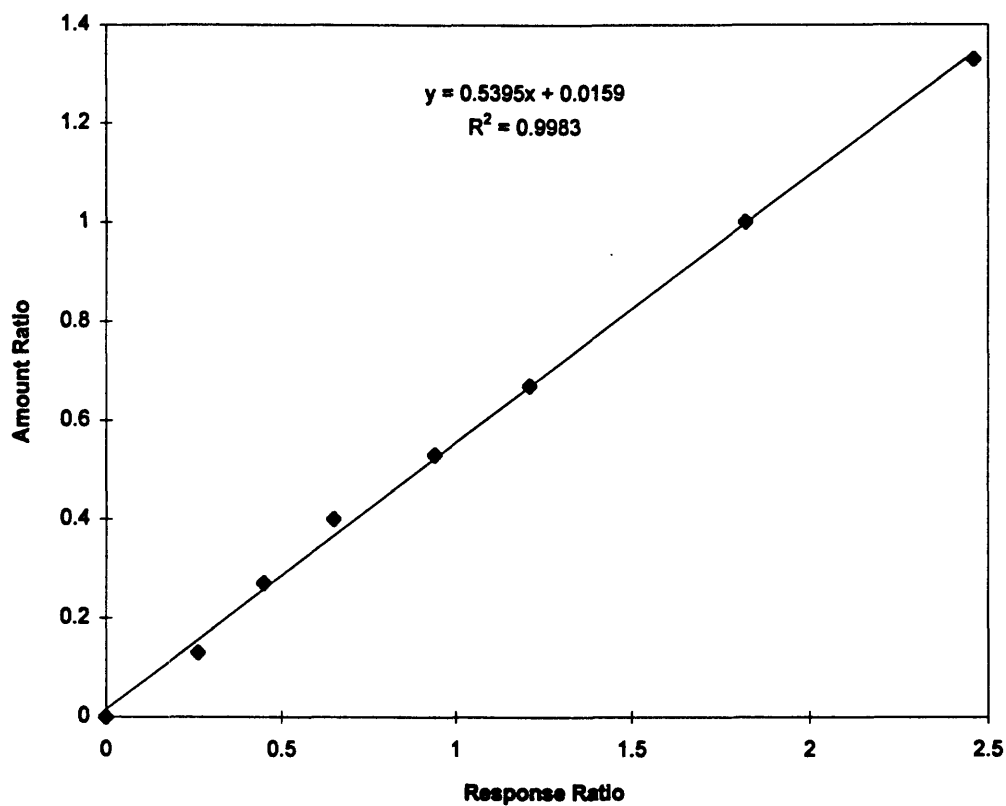


Figure 7. The calibration curve relating 8-oxoG to N<sup>2</sup>-methyl-8-oxoG. 200 pg to 2 ng of 8-oxoG and 1500 pg N<sup>2</sup>-methyl-8-oxoG were quantitated by integration of chromatogram peaks. The amount ratios represent (pg 8-oxoG)/(pg N<sup>2</sup>-methyl-8-oxoG) and the response ratios represent (area of 8-oxoG)/(area of N<sup>2</sup>-methyl-8-oxoG). The curve was linear with a correlation coefficient of 0.998.

varying concentrations of ONOO<sup>-</sup> for 30 minutes at room temperature. The number of strand breaks (Figure 8) and 8-oxoG (Figure 9) in 10<sup>6</sup> bases were determined for different concentrations of ONOO<sup>-</sup> (Table 1). Due to limitations in the sensitivity of the 8-oxoG analysis, levels could not be studied at lower concentrations of ONOO<sup>-</sup>. The number of strand breaks and 8-oxoG in 10<sup>6</sup> bases were then compared at different concentrations (Figure 10) and the ratios of 8-oxoG to strand breaks were calculated, demonstrating different relative levels of 8-oxoG and strand breaks at different concentrations of peroxyntirite.

### **Copper(II)**

Because copper(II) in the presence of hydrogen peroxide has been shown to result in the formation of 8-oxoG (37) and single strand breaks (1, 34) Cu(II) and H<sub>2</sub>O<sub>2</sub> were used as an oxygen radical generating system. 30 µg/mL pUC19 plasmid DNA was treated with different concentrations of Cu(II) and 1 mM H<sub>2</sub>O<sub>2</sub> at room temperature for 30 minutes. The number of strand breaks (Figure 11) and 8-oxoG (Figure 12) per 10<sup>6</sup> bases were quantitated for different concentrations of Cu(II) (Table 2). The amount of strand breaks exceeded single-hit kinetics (1 nick per plasmid which corresponds to ~ 37 strand breaks in 10<sup>6</sup> bases (10)) for most concentrations of Cu(II), so the calculations were outside the specificity of the assay. Although the number of strand breaks in 10<sup>6</sup> bases exceeded single hit kinetics for most concentrations, strand breaks and 8-oxoG per 10<sup>6</sup> bases were compared (Figure 13) and the ratios of 8-oxoG to strand breaks were determined. These ratios show as much as an approximate 2-fold difference in the relative levels of strand breaks and 8-oxoG at different concentrations of

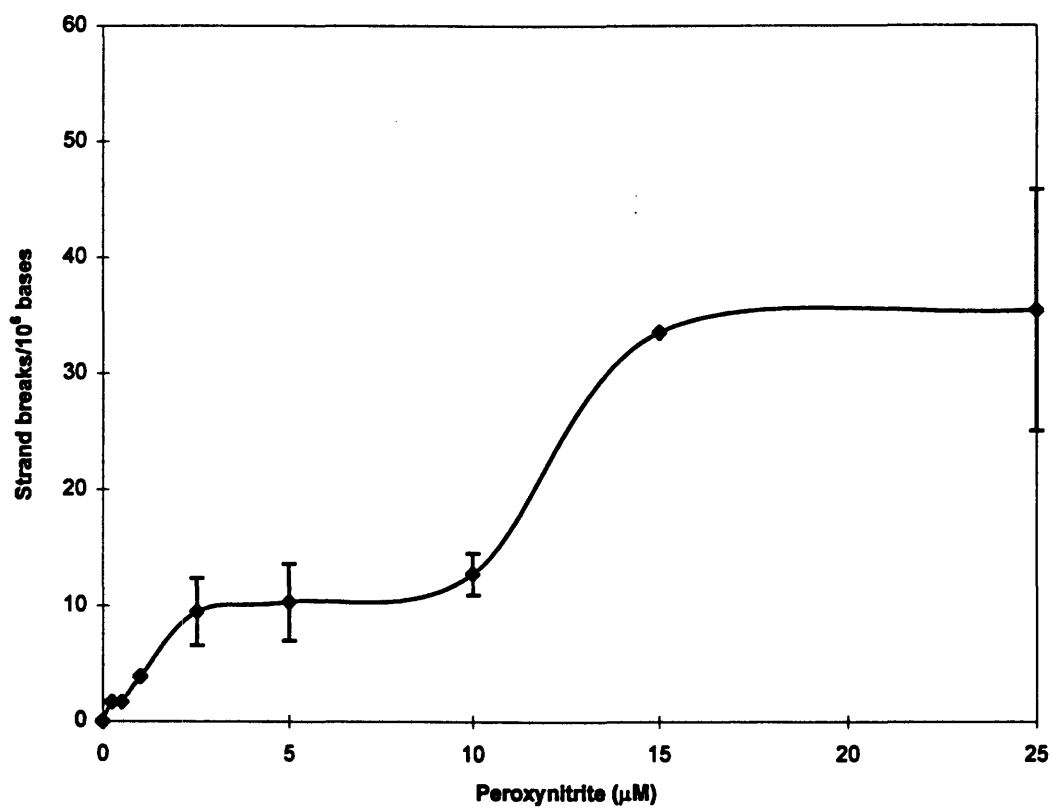


Figure 8. The number of strand breaks in  $10^6$  bases after reaction with  $\text{ONOO}^-$ . 30  $\mu\text{g/mL}$  pUC19 DNA was treated with 0, 0.25, 0.5, 1, 2.5, 5, 10, 15 and 25  $\mu\text{M}$   $\text{ONOO}^-$  at room temperature for 30 minutes. The number of strand breaks was determined by agarose gel electrophoresis followed by phosphorimager analysis.

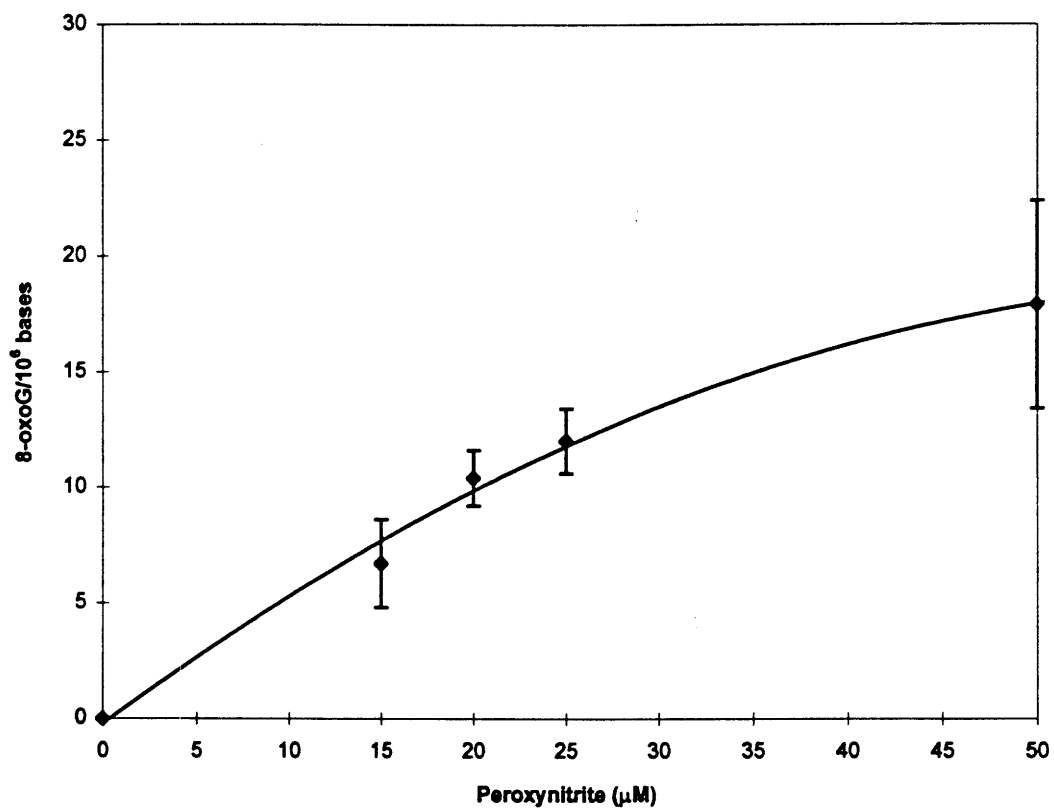


Figure 9. The number of 8-oxoG in  $10^6$  bases after reaction with  $\text{ONOO}^-$ . 30  $\mu\text{g/mL}$  pUC19 DNA was reacted with 0, 15, 20, 25 and 50  $\mu\text{M}$   $\text{ONOO}^-$  at room temperature for 30 minutes in 50 mM potassium phosphate, pH 7.4, Chelexed. The number of 8-oxoG was determined by HPLC-ECD.

Table 1. The number of 8-oxoG and strand breaks in  $10^6$  bases after exposure to different concentrations of peroxynitrite.

[ONOO-]	8-oxoG	n	Strand breaks	n	Ratio
0 $\mu$ M	0		0		
0.25 $\mu$ M	-		1.68	1	
0.5 $\mu$ M	-		1.68	1	
1 $\mu$ M	-		3.91	1	
2.5 $\mu$ M	-		9.51 $\pm$ 2.05	3	
5 $\mu$ M	-		10.40 $\pm$ 2.33	3	
10 $\mu$ M	-		12.90 $\pm$ 1.79	3	
15 $\mu$ M	6.71 $\pm$ 1.92	6	33.51	1	0.20
20 $\mu$ M	10.40 $\pm$ 1.21	3	-		
25 $\mu$ M	12.00 $\pm$ 1.40	5	35.45 $\pm$ 10.43	4	0.34
50 $\mu$ M	17.90 $\pm$ 4.49	3	41.06 $\pm$ 3.26	3	0.44

Where n represents the number of replications of 8-oxoG and strand break quantitation at each concentration of ONOO $\cdot$ .

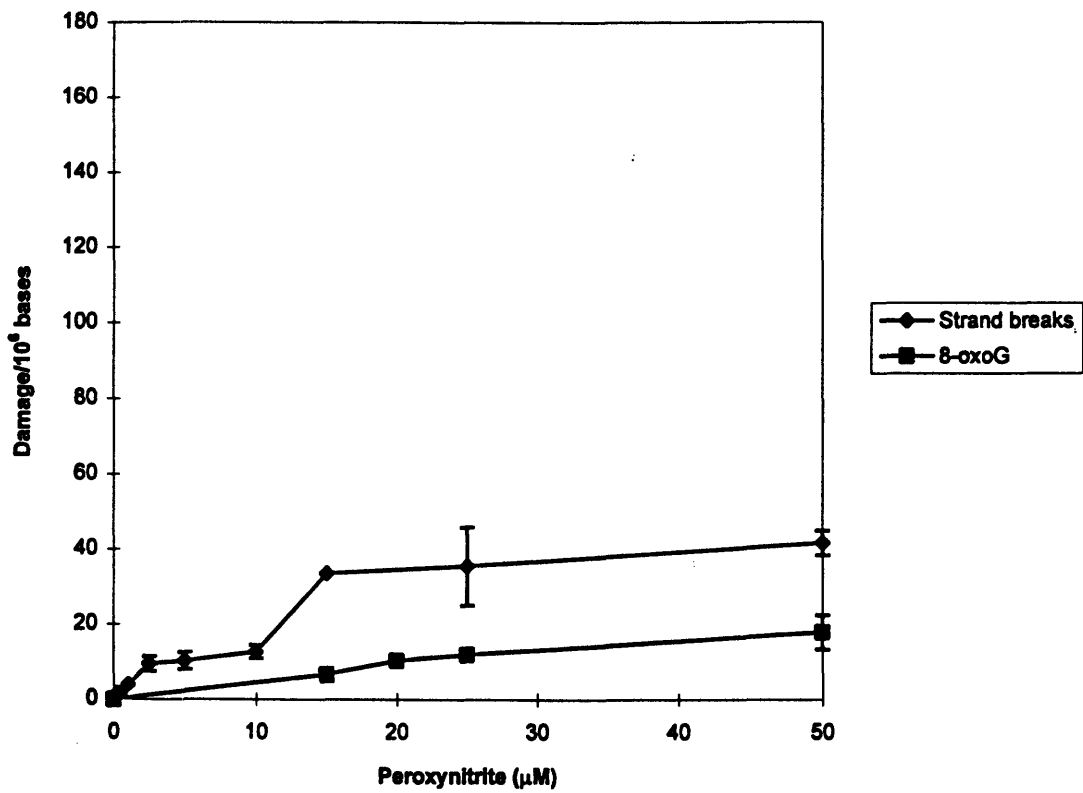


Figure 10. The comparison of 8-oxoG and strand breaks in  $10^6$  bases for different concentrations of  $\text{ONOO}^-$ .

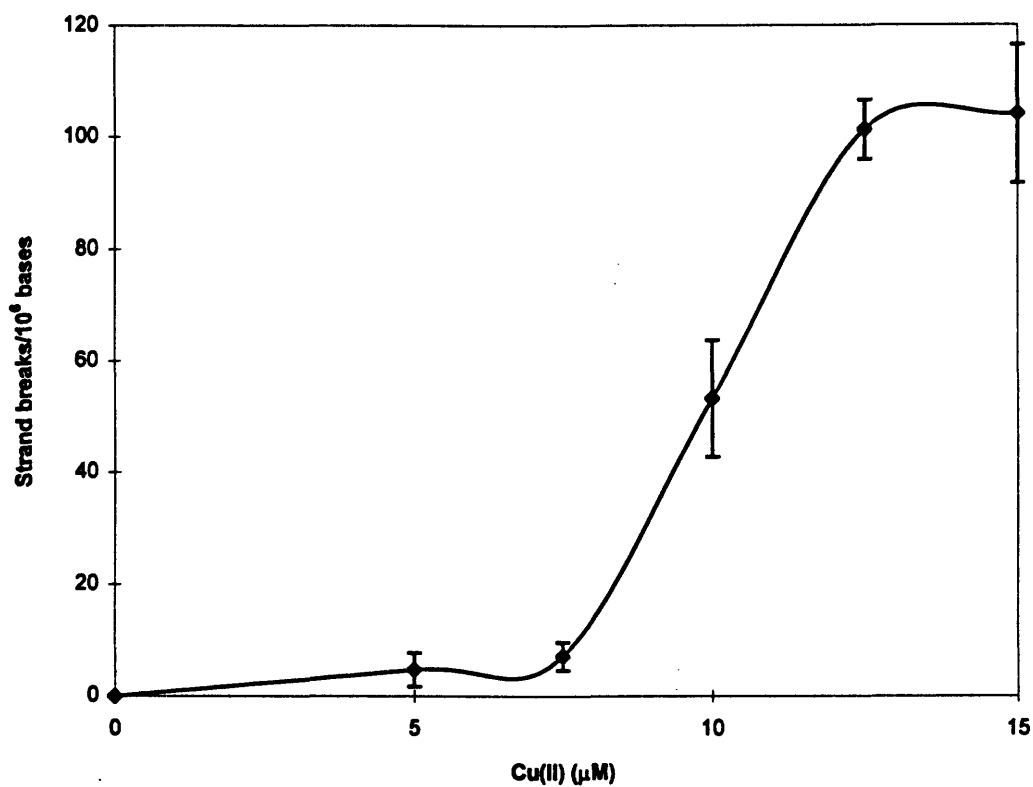


Figure 11. The number of strand breaks in 10<sup>6</sup> bases after treatment with Cu(II) and H<sub>2</sub>O<sub>2</sub>. 30 μg/mL pUC19 DNA was reacted with 0, 5, 7.5, 10, 12.5 and 15 μM Cu(II) and 1 mM H<sub>2</sub>O<sub>2</sub> for 30 minutes at room temperature in 50 mM potassium phosphate, pH 7.4, Chelexed. 10-fold excess EDTA was then added. The number of strand breaks was determined by agarose gel electrophoresis and phosphorimager analysis.

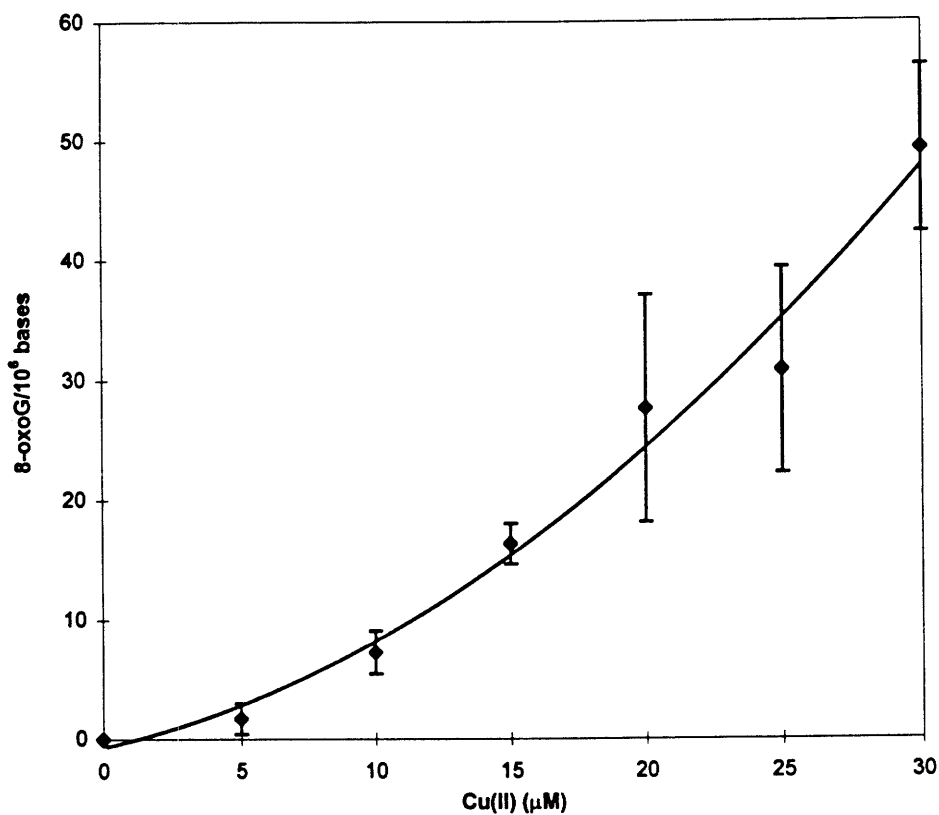


Figure 12. The number of 8-oxoG in  $10^6$  bases after reaction with Cu(II) and  $\text{H}_2\text{O}_2$ . 30  $\mu\text{g/mL}$  pUC19 DNA was reacted with 0, 5, 10, 15, 20, 25 and 30  $\mu\text{M}$  Cu(II) and 1 mM  $\text{H}_2\text{O}_2$  for 30 minutes at room temperature in 50 mM potassium phosphate, pH 7.4, Chelexed. 10-fold excess EDTA was then added. The number of 8-oxoG was determined by HPLC-ECD.

Table 2. The number of 8-oxoG and strand breaks in  $10^6$  bases following treatment with different concentrations of Cu(II) and 1 mM  $H_2O_2$ .

[Cu(II)]	8-oxoG	n	Strand breaks	n	Ratio
0 $\mu$ M	0		0		
5 $\mu$ M	$1.73 \pm 1.32$	3	$4.72 \pm 3.02$	3	0.37
7.5 $\mu$ M	-		$7.17 \pm 2.50$	2	
10 $\mu$ M	$7.27 \pm 1.85$	3	$53.18 \pm 10.49$	3	0.14
12.5 $\mu$ M	-		$101.27 \pm 5.27$	2	
15 $\mu$ M	$16.30 \pm 1.66$	3	$104.12 \pm 12.38$	3	0.17
20 $\mu$ M	$27.60 \pm 9.48$	6	112.06	1	0.25
25 $\mu$ M	$30.80 \pm 8.60$	6	129.56	1	0.24
30 $\mu$ M	$49.30 \pm 7.02$	5	170.14	1	0.30

Where n represents the number of replications of 8-oxoG and strand break quantitation at each concentration of Cu(II).

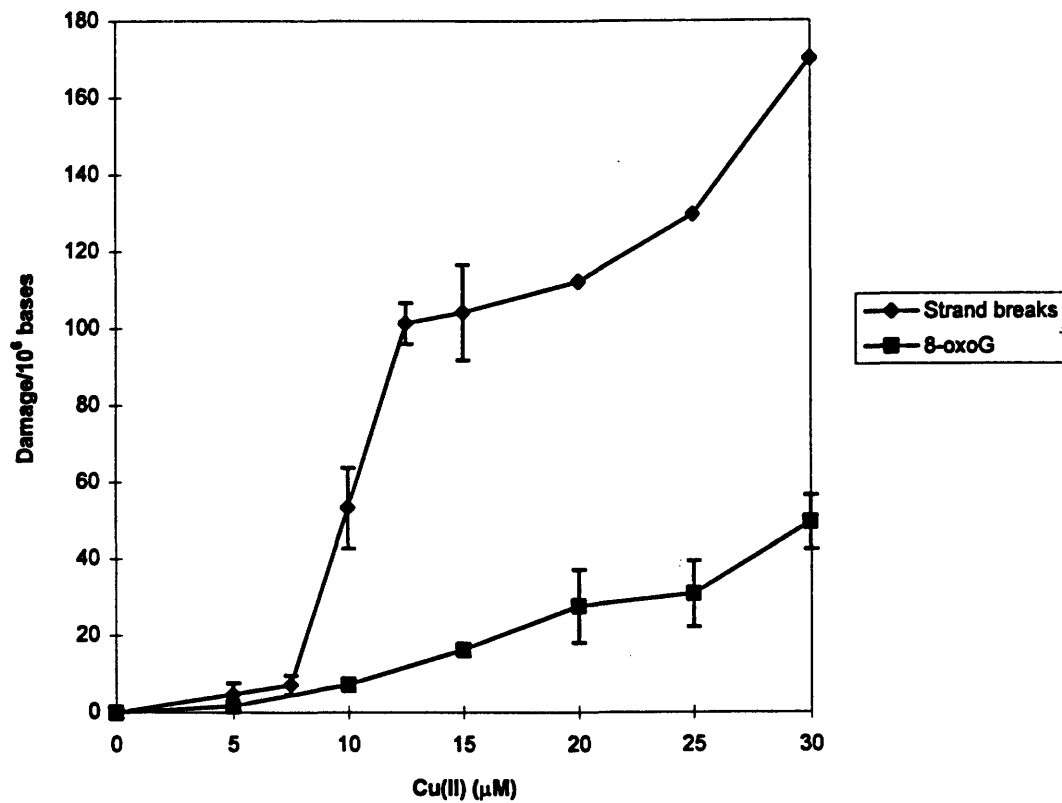


Figure 13. The comparison of 8-oxoG and strand breaks in 10<sup>6</sup> bases for different concentrations of Cu(II) and 1 mM H<sub>2</sub>O<sub>2</sub>.

Cu(II).

### **Iron(II)-EDTA**

We decided to study the effects of iron(II)-EDTA and hydrogen peroxide because the reaction has been proposed to generate hydroxyl radicals and has been shown to induce single strand breaks in DNA without base specificity (39, 40, 49). 30  $\mu\text{g/mL}$  pUC19 plasmid DNA was reacted with varying concentrations of Fe(II) with 2-fold excess EDTA and 1 mM  $\text{H}_2\text{O}_2$  for 30 minutes at room temperature in 50 mM potassium phosphate, pH 7.4, Chelexed. The number of strand breaks (Figure 14) and 8-oxoG (Figure 15) in  $10^6$  bases was determined over a range of Fe(II) concentrations (Table 3). Again, the number of 8-oxoG could not be determined at lower concentrations of Fe(II) due to limitations in the sensitivity of the assay. The number of 8-oxoG and strand breaks in  $10^6$  bases were compared (Figure 16) and the ratios of 8-oxoG to strand breaks were calculated.

### **Gamma radiation.**

Ionizing radiation is known to produce hydroxyl radicals through the homolytic fission of oxygen-hydrogen bonds in water. Gamma radiation has been demonstrated to form single strand breaks (13, 34, 50) and 8-oxoG in DNA (42) and therefore was used as an oxidizing agent. 30  $\mu\text{g/mL}$  pUC19 plasmid DNA in 50 mM potassium phosphate, pH 7.4, Chelexed, both air and  $\text{N}_2\text{O}$  saturated, was exposed to different levels of radiation from a  $^{60}\text{Co}$   $\gamma$  source. Slightly higher levels of strand breaks were observed under  $\text{N}_2\text{O}$  versus air saturation, and no significant effect was observed for 8-oxoG. The data reported are for air saturated solutions. The number of strand breaks (Figure 17) and

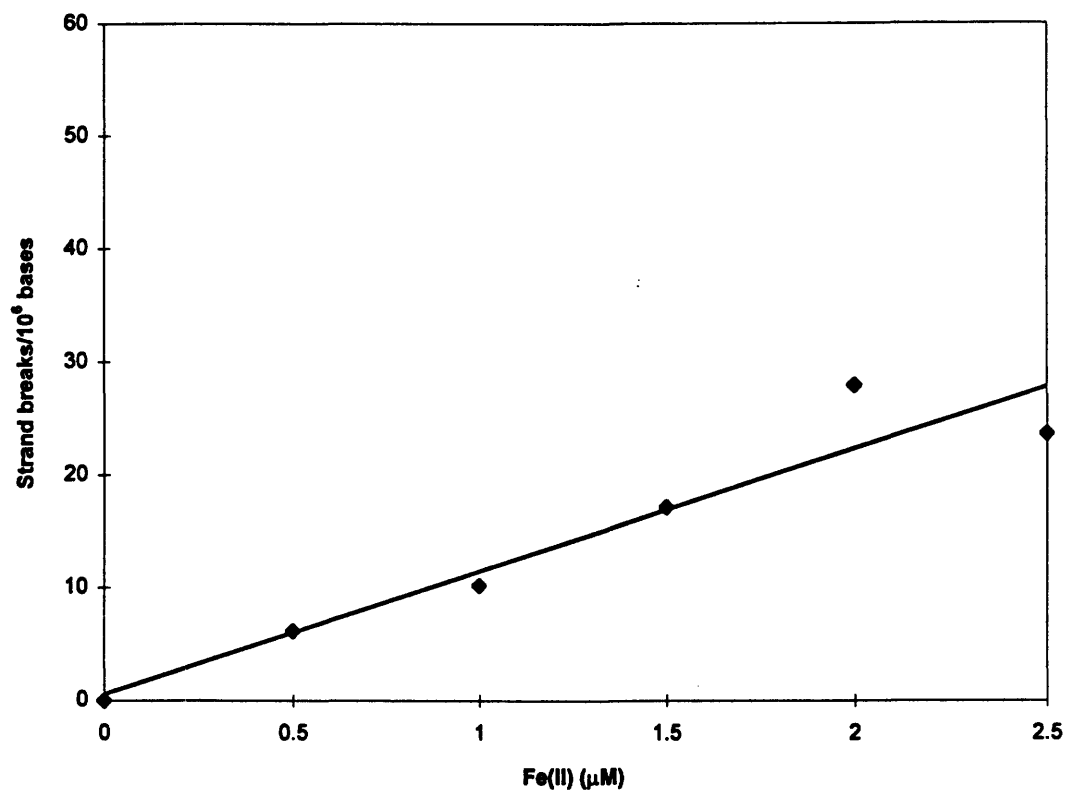


Figure 14. The number of strand breaks in  $10^6$  bases after treatment with Fe(II)-EDTA and  $\text{H}_2\text{O}_2$ . 30  $\mu\text{g}/\text{mL}$  pUC19 DNA was reacted with 0, 0.5, 1, 1.5, 2 and 2.5  $\mu\text{M}$  Fe(II) with 2-fold excess EDTA and 1 mM  $\text{H}_2\text{O}_2$  for 30 minutes at room temperature in 50 mM potassium phosphate, pH 7.4, Chelexed. The number of strand breaks was determined by agarose gel electrophoresis and phosphorimager analysis.

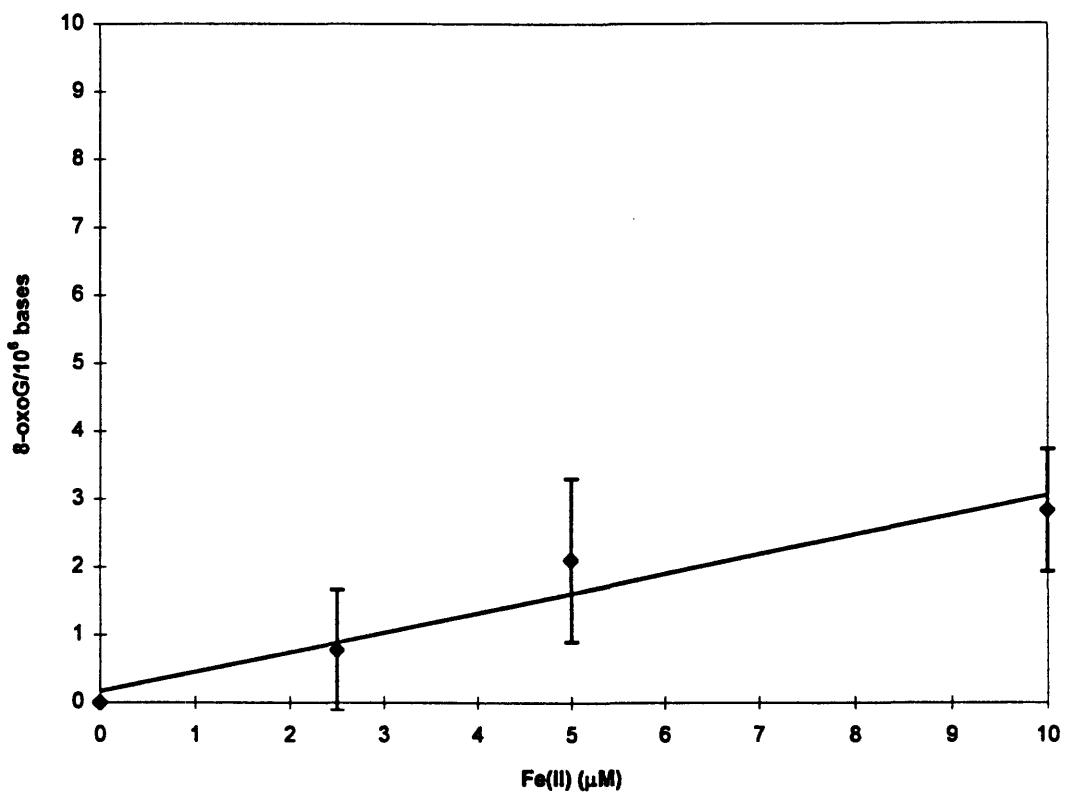


Figure 15. The number of 8-oxoG in 10<sup>6</sup> bases after treatment with Fe(II)-EDTA and H<sub>2</sub>O<sub>2</sub>. 30 μg/mL pUC19 DNA was reacted with 0, 2.5, 5 and 10 μM Fe(II) with 2-fold excess EDTA and 1 mM H<sub>2</sub>O<sub>2</sub> for 30 minutes at room temperature in 50 mM potassium phosphate, pH 7.4, Chelexed. The number of 8-oxoG was determined by HPLC-ECD.

Table 3. The number of 8-oxoG and strand breaks in  $10^6$  bases after treatment with different concentrations of Fe(II) and 1 mM  $H_2O_2$ .

[Fe(II)]	8-oxoG	n	Strand breaks	n	Ratio
0 $\mu$ M	0		0		
0.5 $\mu$ M	-		6.14	1	
1.0 $\mu$ M	-		10.24	1	
1.5 $\mu$ M	-		17.13	1	
2.0 $\mu$ M	-		27.92	1	
2.5 $\mu$ M	$0.78 \pm 0.89$	6	$23.58 \pm 5.98$	3	0.03
5.0 $\mu$ M	$2.10 \pm 1.25$	6	$62.11 \pm 10.43$	3	0.03
10.0 $\mu$ M	$2.83 \pm 0.88$	6	$141.10 \pm 31.60$	3	0.02

Where n represents the number of replications of 8-oxoG and strand break quantitation at each concentration of Fe(II).

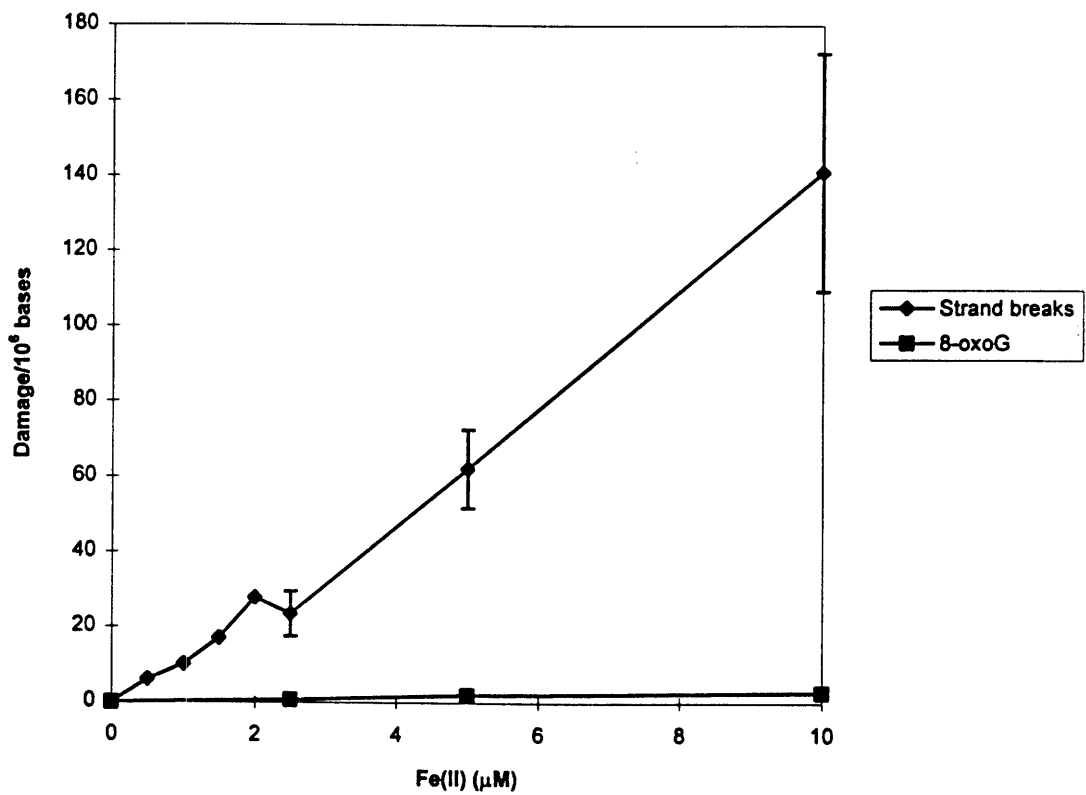


Figure 16. The comparison of 8-oxoG and strand breaks in 10<sup>6</sup> bases for different concentrations of Fe(II) with 2-fold excess EDTA and 1 mM H<sub>2</sub>O<sub>2</sub>.

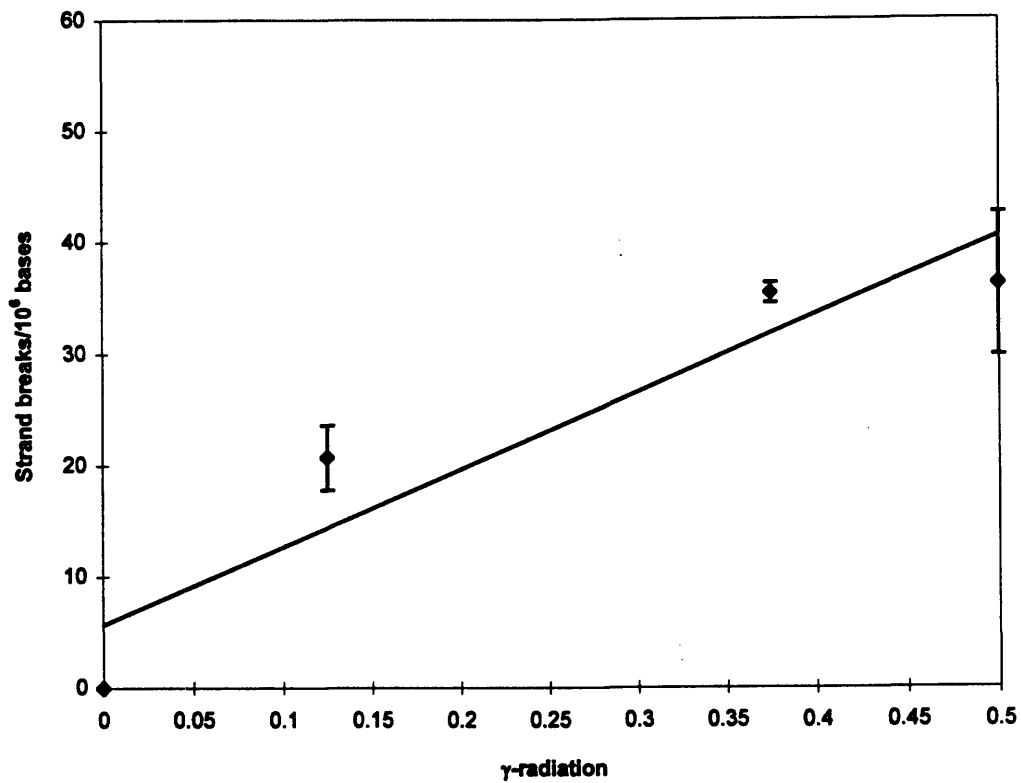


Figure 17. The number of strand breaks in  $10^6$  bases after exposure to different levels of gamma radiation.  $30 \mu\text{g/mL}$  pUC19 DNA in  $50 \text{ mM}$  potassium phosphate, pH 7.4, Chelexed, was exposed to 0, 0.125, 0.375 and 0.5 Gy radiation. The number of strand breaks was determined by agarose gel electrophoresis and phosphorimager analysis.

8-oxoG (Figure 18) in  $10^6$  bases was determined (Table 4). Due to the extensive nicking at higher exposures, the number of strand breaks quantitated by agarose gel electrophoresis is likely an underestimate of the actual number of strand breaks. The number of 8-oxoG and strand breaks were compared for different levels of exposure to radiation (Figure 19) and the ratios of 8-oxoG to strand breaks were calculated.

### **Comparison of Damage from Different Oxidizing Agents**

The number of 8-oxoG in  $10^6$  bases versus the number of strand breaks in  $10^6$  bases was used to compare the relative levels of damage produced by the four oxygen radical generating systems (Figure 20). As shown in Figure 20, the oxidizing agents produced different amounts of 8-oxoG relative to strand breaks. In addition, the relationship of 8-oxoG to strand breaks was dependent on the agent, as demonstrated by the different shapes of the curves. Fe(II)-EDTA and  $\gamma$ -radiation produced similar amounts of 8-oxoG relative to strand breaks and both exhibited a linear relationship between increases in 8-oxoG and in strand breaks. These results indicate that both agents generate a common reactive oxygen species with similar specificity in reactions leading to DNA damage. Cu(II) and ONOO<sup>-</sup> both produced higher levels of 8-oxoG relative to strand breaks than Fe(II)-EDTA and  $\gamma$ -radiation. However, the relationship between increases in 8-oxoG and in strand breaks was different for Cu(II) and ONOO<sup>-</sup> as evidenced by the different shapes of the curves. The increase in 8-oxoG relative to increase in strand breaks was non-linear for Cu(II) indicating an intermediate different from that seen with Fe(II)-EDTA and  $\gamma$ -radiation. For ONOO<sup>-</sup>, the relationship between

8-oxoG and strand breaks appeared to be biphasic which could result from distinct one-electron and two-electron oxidative reactions.

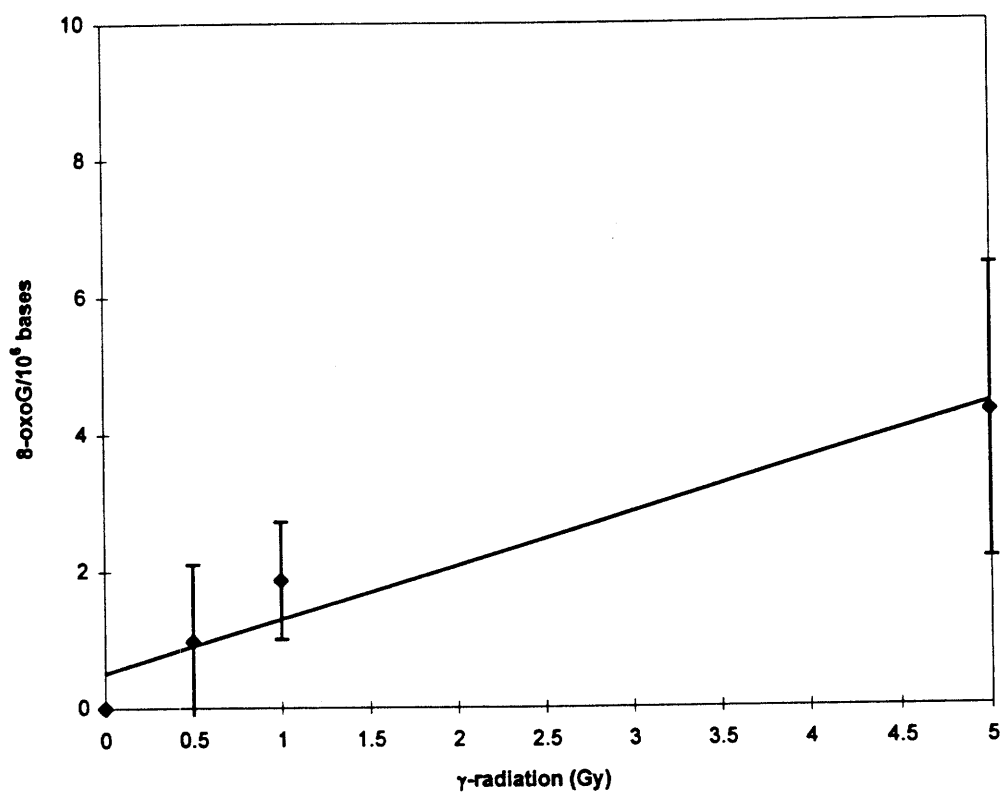


Figure 18. The number of 8-oxoG in  $10^6$  bases following exposure to different amounts of gamma radiation. 30  $\mu\text{g/mL}$  pUC19 DNA in 50 mM potassium phosphate, pH 7.4, Chelexed, was exposed to 0, 0.5, 1 and 5 Gy of radiation. The number of 8-oxoG was determined by HPLC-ECD.

Table 4. The number of 8-oxoG and strand breaks in  $10^6$  bases after exposure to different levels of gamma radiation.

$\gamma$ -rad.	8-oxoG	n	Strand breaks	n	Ratio
0	0		0		
0.125 Gy	-		$20.70 \pm 2.90$	2	
0.375 Gy	-		$35.40 \pm 0.90$	2	
0.5 Gy	$0.97 \pm 1.13$	4	$36.24 \pm 6.43$	3	0.03
1.0 Gy	$1.86 \pm 0.86$	6	$48.90 \pm 8.14$	3	0.04
1.5 Gy	-		$50.80 \pm 12.80$	2	
5.0 Gy	$4.29 \pm 2.14$	5	$90.55 \pm 21.33$	3	0.05
10.0 Gy	-		$107.77 \pm 10.28$	2	

Where n represents the number of replications of 8-oxoG and strand break quantitation at each exposure of  $\gamma$ -radiation.

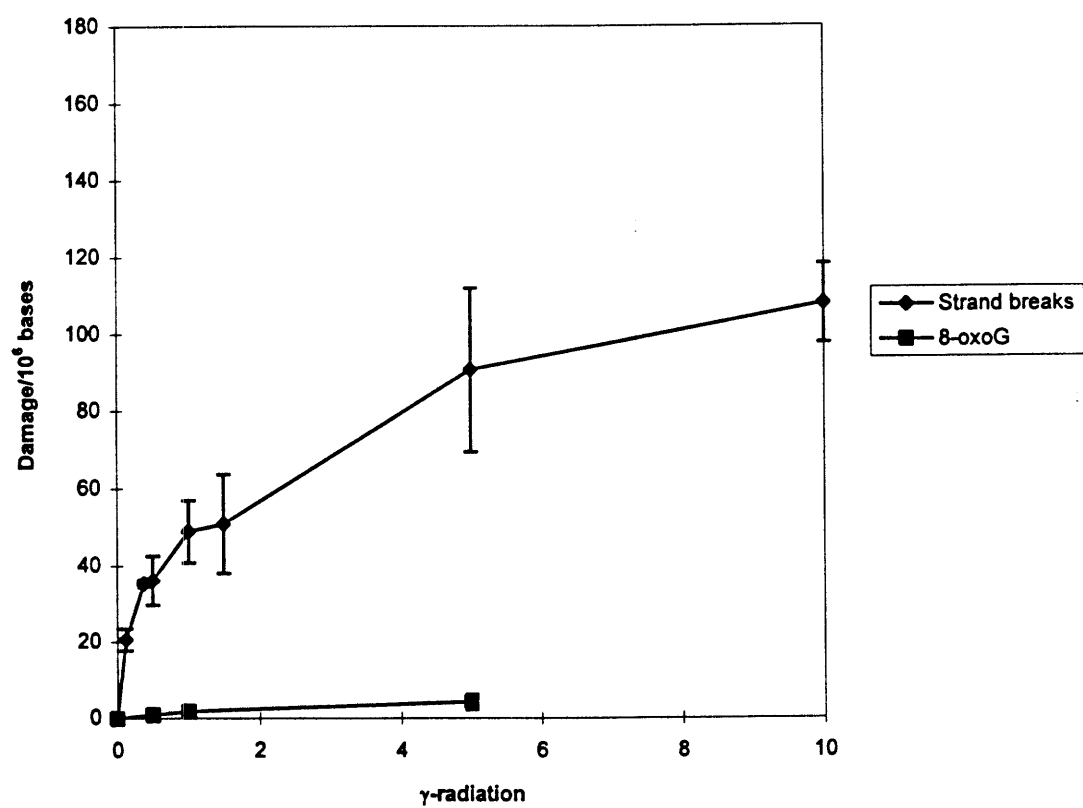


Figure 19. The comparison of 8-oxoG and strand breaks in 10<sup>6</sup> bases following exposure to different levels of gamma radiation.

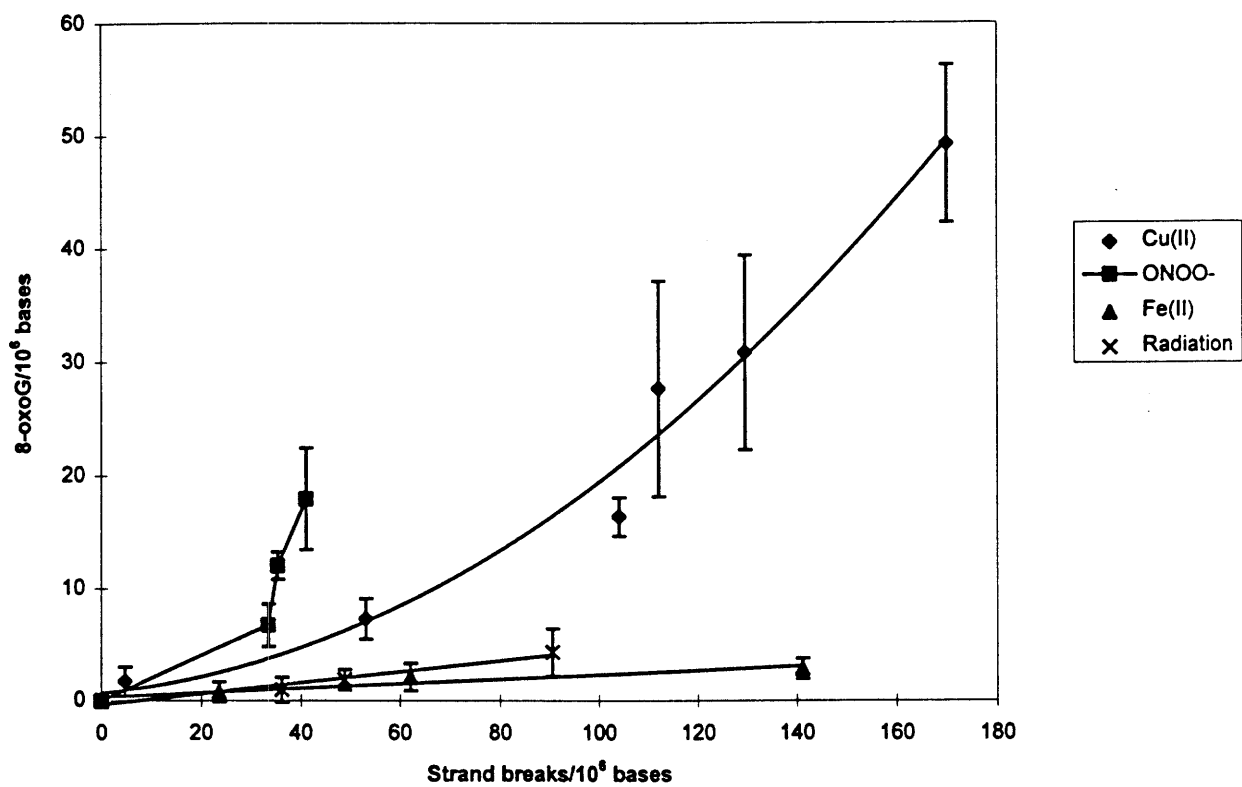


Figure 20. The comparison of relative levels of 8-oxoG and strand breaks in  $10^6$  bases for peroxynitrite, copper(II) and hydrogen peroxide, iron(II)-EDTA and hydrogen peroxide, and gamma radiation.

## DISCUSSION

Oxidative damage resulting from exposure to reactive oxygen species is believed to play a significant role in mutagenesis, aging and carcinogenesis (51). Numerous DNA damage products have been detected after exposure of DNA to oxidizing agents. Types of oxidative DNA damage include single and double strand breaks, abasic sites, modified bases and DNA-protein crosslinks. At least 17 different modified base products involving all four DNA bases have been associated with oxidative damage, including thymine glycol, cytosine glycol, 8-oxoadenine and 8-oxoguanine (6). Of these, 8-oxoG is most commonly used as a biomarker due to the high levels formed and the availability of a sensitive analytical technique (3). Most studies of specific oxygen radical generating systems use a single oxidative DNA damage product as an index of total oxidative damage.

Our results challenge the use of a single biomarker in investigations of oxidative damage when comparing more than one oxidizing agent or in studies of a single agent at different concentrations. We have demonstrated that the relative levels of 8-oxoG and strand breaks, two of the most commonly used biomarkers of oxidative damage, are not equal for different oxygen radical generating systems and can vary by more than an order of magnitude depending on the system. The relative levels can also vary between different concentrations of a single agent as shown most dramatically by Cu(II) and H<sub>2</sub>O<sub>2</sub>. Our results indicate that different reactive oxygen species and/or binding specificities can contribute to different levels and types of oxidative damage to DNA.

Our data for the relative amounts of oxidative damage formed by Fe(II)-EDTA with hydrogen peroxide and gamma radiation support the hypothesis that a common intermediate is involved in each system. Both agents generated similar levels of 8-oxoG relative to strand breaks and also demonstrated a linear relationship between increasing 8-oxoG and increasing strand breaks. This result is in good agreement with previous studies of Fe(II)-EDTA which compared the specificity of DNA damage to that produced by gamma radiation (39, 40). Tullius has found that the pattern of single strand breaks produced by Fe(II)-EDTA is identical to that of gamma radiation (39) and Chiu, et al. has found similar patterns for double strand breaks with the agents in isolated nuclear chromatin (29).

Both Fe(II)-EDTA and gamma radiation are proposed to generate hydroxyl radicals which lead to oxidative DNA damage. The significantly higher levels of strand breaks relative to 8-oxoG which we observed are consistent with this mechanism. Hydroxyl radicals will react without sequence specificity and studies have estimated that 10 to 20% of attacks by the hydroxyl radical occur at the deoxyribose with the remaining attacks occurring on the base (13). However, the C-8 position of guanine is just one of numerous potential sites of attack at the base if the hydroxyl radical reacts without base sequence specificity. Thus, the overall probability of hydroxyl radical attack giving rise to 8-oxoG is significantly less than that of single strand breaks.

Unlike Fe(II)-EDTA and gamma radiation, Cu(II) with hydrogen peroxide did not produce a linear relationship between increases in 8-oxoG and increases in strand breaks. In addition, the levels of 8-oxoG relative to strand breaks were significantly higher with

Cu(II) than the Fe(II)-EDTA and radiation systems. This difference could be due to a different reactive oxygen species and/or the binding specificity of Cu(II) to DNA. Studies have found Cu(II) binds covalently to DNA through the N-7 of guanine (30, 31). If Cu(II) were to bind DNA and then react with hydrogen peroxide to generate the hydroxyl radical, the radicals would attack the DNA in the immediate vicinity of the bound Cu(II) because the radical is highly reactive and would react before diffusion could occur. This site specific mechanism could explain the higher levels of 8-oxoG relative to strand breaks observed with Cu(II) because the C-8 position of guanine would be more susceptible than other base sites to hydroxyl radical attack. This mechanism has been supported by a partial protection of DNA in studies with hydroxyl radical scavengers indicating that hydroxyl radicals are formed and contribute to DNA damage, but the efficiency of scavengers is blocked by the site specific reaction (1). In addition, the spectrum of base damage resulting from Cu(II) and hydrogen peroxide is similar to that of hydroxyl radical-induced DNA damage (7, 37).

However, another possible mechanism would involve some other reactive oxygen species being generated which also reacts with site specificity. Yamamoto and Kawanishi have proposed that a copper-peroxide compound is the agent responsible for the oxidative damage observed with Cu(II) (52). According to the mechanism, Cu(II) binds DNA and two adjacently bound Cu(II) ions then react with hydrogen peroxide to form a copper-peroxide compound which ultimately reacts with the DNA. This mechanism would also be consistent with higher levels of 8-oxoG relative to strand breaks.

An additional anomaly with Cu(II) is the shape of the curve relating the number of strand breaks in  $10^6$  bases to increasing concentrations of Cu(II). The sigmoidal shape of the strand break curve could be indicative of a cooperative effect. One possible explanation is that the binding of Cu(II) to DNA is enhanced with increasing concentrations of Cu(II). Perhaps the binding of an initial Cu(II) ion facilitates the binding of additional Cu(II) ions which would result in higher numbers of strand breaks as the concentration of Cu(II) increases. Another possibility is the participation of Cu(I) in the reaction. Cu(I) is unstable and readily oxidized to Cu(II), but could exist as an impurity in Cu(II) solutions. Studies with bathocuproine, a Cu(I)-specific chelating agent, have found Cu(I) to be involved in DNA damage (1, 52). A cooperative effect could exist in the conversion of Cu(II) to Cu(I) resulting in additional Cu(I) at increasing levels of Cu(II). Cu(I) has been shown to damage DNA with much less site specificity than Cu(II) (1) which could explain why only strand breaks exhibit the cooperative effect. However, many other possible explanations exist due to the complex chemistry of Cu(II). The reaction of Cu(II) and  $H_2O_2$  with DNA would have to be studied further before anything could be conclusively determined.

Regardless of the actual mechanism, 8-oxoG levels rose gradually and did not exhibit an increase similar to the levels of strand breaks. This difference is demonstrated by the decrease in the ratio of 8-oxoG to strand breaks by more than a factor of 2 between 5 and 10  $\mu$ M Cu(II). These results clearly show that the chemistry leading to the formation of strand breaks versus 8-oxoG is not the same over that range of Cu(II) concentrations.

Rodriguez, et al. also examined base damage relative to strand breaks in copper and hydrogen peroxide-induced DNA damage and found the ratio of endonuclease-sensitive base lesions to strand breaks to be 3.4 which is more than 10-fold higher than the ratios we determined (5). One potential reason for this difference is the use of ascorbate as a reducing agent by Rodriguez, et al. Addition of ascorbate to the Cu(II) and hydrogen peroxide system has been shown to significantly increase the amount of base damage (7). Another difference in the Rodriguez study was the use of Nth and Fpg proteins to identify the oxidatively damaged bases. Unlike the HPLC-ECD assay which we used, the Nth and Fpg proteins are not specific for 8-oxoG, but rather identify most oxidatively damaged pyrimidine and purine bases, respectively. Finally, we treated the DNA with putrescine prior to analysis so the number of strand breaks observed included abasic sites, whereas Rodriguez, et al. only detected single strand breaks existing after the reaction with Cu(II). The differences between the studies could easily explain the 10-fold higher ratio reported by Rodriguez, et al. over the ratios which we determined for copper and hydrogen peroxide-induced DNA damage. Although the ratio of base damage to strand breaks was higher, Rodriguez, et al. did find the damage to be nonrandom and sequence-dependent through mapping of the induced damage which supports the site-specific nature of DNA base damage related to the binding of Cu(II).

Peroxynitrite also generated higher levels of 8-oxoG relative to strand breaks when compared with Fe(II)-EDTA and gamma radiation. However, the higher ratios were due largely in part to fewer strand breaks being formed in addition to increased levels of 8-oxoG. This difference is likely due to the reactive oxygen species involved in

DNA damage. Peroxynitrite has been proposed to react via either a caged radical mechanism, involving  $\cdot\text{OH}$  and  $\cdot\text{NO}_2$ , or through a high energy intermediate,  $\text{HOONO}^*$  (25). The reactivity of peroxynitrite has been studied in depth and currently the high energy intermediate mechanism is favored to explain data from scavenger studies in which hydroxyl radical scavengers are incapable of completely blocking nitrate formation or the oxidation of substrates (23). The high energy intermediate is also favored in thermodynamic and quantum mechanical calculations (45).

When the level of either 8-oxoG or strand breaks in  $10^6$  bases is plotted against increasing concentrations of peroxynitrite, the plot is curved. This result is consistent with data presented by Pryor, et al. (25). This curvature is used to provide additional support for the  $\text{HOONO}^*$  mechanism versus the hydroxyl radical mediated reaction. In their studies, the extent of the curvature was dependent on the substrate reactivity and concentrations of the substrate used. Our studies are slightly different in that we varied the concentration of peroxynitrite while fixing the concentration of substrate, but the results are still comparable.

The number of strand breaks in  $10^6$  bases was determined at lower concentrations of peroxynitrite to more closely examine the shape of the curve. In addition to the overall curvature, two distinct curves can be detected with one below  $2.5 \mu\text{M}$  and the other above  $5 \mu\text{M}$  peroxynitrite which support the presence of a biphasic reaction. Peroxynitrite has been shown to react with substrates in both a one-electron oxidation and a two-electron reaction (25). However, these mechanisms are difficult to distinguish because both reactions can give rise to the same products. Pryor, et al. was able to separate and study

the individual mechanisms through the reaction of peroxynitrite with methionine because different products were generated (25). The one-electron oxidation involves an electron transfer (ET) and is first-order with respect to HOONO\* and zero-order in substrate. The two-electron oxidation is a SN2 reaction and is second-order overall, being first-order with respect to both HOONO and substrate (23).

Due to the presence of two distinct curves, our data is consistent with a system of two reactions which ultimately generate the same products. As described by Pryor, the ET reaction could occur through a mechanism of hydrogen atom abstraction that would lead to strand break formation. The SN2 mechanism would involve nucleophilic attack on the peroxide of HOONO causing the addition of [OH] to the deoxyribose. Rearrangement could then generate strand breaks. All the 8-oxoG data represents the single reaction which occurs at higher concentrations of ONOO<sup>-</sup> because the analysis is not sensitive enough to detect 8-oxoG at the low concentrations of ONOO<sup>-</sup> where the two reactions are distinct.

## CONCLUSION

In conclusion, we have demonstrated that oxidative DNA damage is a function of the oxidizing agent and its concentration. As a result, the chemistry of the oxygen radical generating system must be considered when studying oxidative damage. We showed that Fe(II)-EDTA/H<sub>2</sub>O<sub>2</sub> and gamma radiation produced similar levels of 8-oxoG relative to strand breaks indicating that both agents react through a common intermediate, probably the hydroxyl radical. Cu(II)/H<sub>2</sub>O<sub>2</sub> and peroxyxynitrite both produced higher ratios of 8-oxoG to strand breaks relative to Fe(II)-EDTA and gamma radiation. However, the differences in the nature of the relationship of 8-oxoG and strand breaks as evidenced by the shapes of curves relating increasing 8-oxoG to increasing strand breaks indicates that Cu(II) and peroxyxynitrite do not react via a common pathway. The higher levels observed with Cu(II) can be explained by a site specific mechanism whereas a reactive oxygen species with greater specificity than the hydroxyl radical could result in the higher levels generated by peroxyxynitrite. Overall, these results have implications in the use of a single biomarker for monitoring oxidative DNA damage arising from different oxidizing agents or different concentrations of a single agent.

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