

**A Study of the Deoxyribose Fragmentation Products Formed by
Oxidation of DNA with γ -Radiation, Peroxynitrite and
Calicheamicin**

by

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In memory of
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in Chemistry

Abstract

Reactive oxygen species, produced endogenously or by exposure to environmental chemicals and ionizing radiation, induce a wide range of DNA lesions. The products resulting from direct oxidation of the deoxyribose sugar have been extensively characterized *in vitro*. Moreover, the chemical environment of cellular DNA is postulated to play a direct role in the chemistry of radical-mediated damage. Here glutathione, a major thiol in mammalian cells, is shown to inhibit the formation of 3'-phosphoglycolate in an *in vitro* model involving γ -radiation of a plasmid restriction fragment. To further assess the role of glutathione in oxidative DNA damage, comparisons of the C4'-chemistries induced by calicheamicin γ_1^I and a structural analogue, calicheamicin θ_1^I , were made. The unique activation of calicheamicin θ_1^I , which unlike calicheamicin γ_1^I does not require thiols, provides an opportunity to determine the effects of thiols on the formation of these DNA damage products.

In addition to studying the effects of glutathione on oxidative DNA damage, the relative level of strand breaks *versus* base damage was investigated for four oxidizing agents. The premise for these studies is that the variety of chemical mechanisms associated with different oxidants should result in a unique spectrum of DNA damage products. To test this hypothesis, both strand breaks and 8-oxoguanine (8-oxoG) were measured in DNA after exposure to γ -radiation, Fe(II)-EDTA/H₂O₂, Cu(II)/H₂O₂, and peroxynitrite at concentrations approaching physiological relevance. The ratio of 8-oxoG to strand breaks varied more than 10-fold depending on the oxidizing agent: ~ 0.4 for peroxynitrite and Cu(II)/H₂O₂ and ~ 0.03 for Fe(II)-EDTA/H₂O₂ and γ -radiation. The level of 8-oxoG relative to strand breaks produced by peroxynitrite was higher than that produced by Fe(II)-EDTA/H₂O₂ and γ -radiation, which is consistent with the altered reactivity or accessibility of a non-hydroxyl radical species produced by peroxynitrite.

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Introduction

DNA strand breaks have been studied both *in vitro* and *in vivo* and their quantitation has been used as an index of the overall DNA damage and repair occurring in cells [1]. Most evidence favors a role for radical-mediated oxidative DNA damage in the etiology of cancer and in cell death [2-4]. In general, bistranded lesions appear to correlate best with the cytotoxicity associated with oxidizing DNA-damaging agents [5]. Furthermore, available evidence indicates that the structure of double-strand lesions affects the ability of cells to repair DNA damage. For example, an abasic site opposite a strand break has been shown to be repaired less efficiently by both exonucleases and endonucleases when compared to damage consisting of an abasic site alone [6-11]. However, due to the multitude of different DNA lesions that are produced, the biological effects of individual lesions have proven difficult to assess.

Oxidation of DNA by γ -irradiation, bleomycin (BLM), transition metal cations/hydrogen peroxide complexes, the enediyne anti-tumor antibiotics and other genotoxic agents occurs *via* radical-mediated processes [12-16]. These agents induce specific types of deoxyribose sugar damage including oxidized apurinic/apyrimidinic (abasic) sites. Characterization of these products and putative intermediates has provided insights into both the mechanism of their formation and their biological significance [12-16]. Information about these radical-mediated DNA damaging events have proven important for understanding the role of these products in the aging process and in neoplastic diseases.

Radical-mediated oxidation of deoxyribose produces a variety of products depending on the position at which the damage occurs (reviewed in [15]). Abstraction of the 1'-hydrogen results in the formation of a 2'-deoxyribolactone abasic site [17,18]. Abstraction of the 4'-hydrogen produces either a 4'-

keto-1'-aldehyde abasic site or strand breaks with 3'-phosphoglycolate (3'-PG) and 5'-phosphate ends [14,19]. Bleomycin [12] and calicheamicin [20] are examples of agents that cause C4'-hydrogen abstraction, while damage produced by Fe(II)-EDTA [16] and, as I show in this thesis, peroxyxynitrite consists of 3'-PG residues among other products that are consistent with C4'-chemistry. Finally, 5'-hydrogen abstraction results in a strand break with a 3'-phosphate and a 5'-nucleoside aldehyde [15,21]. From the major groove of DNA, radical-mediated removal of the 3'-hydrogen results in a strand break with a 3'-phosphoglycoaldehyde and a 5'-phosphate [22]. Abstraction of the 2'-hydrogens which sit both in the major and minor grooves of DNA is not energetically favored [23].

While there are many agents that have been found to produce oxidative DNA damage, the mechanisms of product formation appear to differ significantly. For example, deoxyribose fragmentation following initial C4'-hydrogen abstraction can partition along either of two pathways to form an abasic site or a strand break with a 3'-phosphoglycolate residue and base propenal [12,15]. Moreover, neocarzinostatin (NCS) [24], esperamicin A₁ (ESP A₁) [25] calicheamicin γ_1^I (CAL γ_1^I) [20] and C-1027 [26] all produce the 3'-PG residue only as part of a double-stranded lesion, while the same product forms in single-strand breaks produced by BLM [19], γ -radiation [27,28], possibly Fe(II)-EDTA [16] and peroxyxynitrite as shown here in Chapter 1. In one of the proposed mechanisms for the formation of 3'-PG in single-strand breaks resulting from exposure to γ -irradiation, a C4'-oxyradical intermediate is believed to be involved [29]. Reduction of this putative intermediate could lead to the formation of the 4'-keto-1'-aldehyde abasic site [29]. These examples highlight the complexity of our current understanding of the mechanisms by which various oxidizing agents damage DNA.

Recent observations with neocarzinostatin and the related enediynes ESP A₁ and CAL γ_1^I suggest that there is a unique chemical mechanism involved in the formation of double-stranded lesions. In our laboratory, we have observed that 3'-PG, which results from abstraction of the C4'-hydrogen, occurs only in the bistranded lesions induced by NCS and CAL γ_1^I [20,24,30]. Similar observations have been made with the C5'-chemistry produced by NCS: namely, the 3'-phosphate-ended fragments arising from 3'-formylphosphate residues occur at a lower frequency in single-stranded lesions [24,30]. Moreover, the formation of these two products may also occur *via* an oxyradical intermediate [24,30]. This conclusion is based on the appearance of NCS-induced 3'-PG residues in single-stranded breaks and the increased formation of 3'-formylphosphate observed under anaerobic conditions in the presence of a nitroaromatic radiation sensitizer, which presumably reacts with the carbon radical (thereby serving as an oxygen substitute) to form the oxyradical intermediate. [24,31,32].

The observed damage chemistry of bistranded lesions is consistent with an interaction between damage sites on opposite strands of the lesion. To explain this phenomenon, a model has been proposed in which the peroxyradicals at the C4'- and C5'- positions on opposite strands react across the minor groove to form a tetraoxide bridge [24]. Breakdown of the bridge may occur by a Russell-like mechanism involving elimination of molecular oxygen [29]. This results in the formation of alkoxyradicals on each strand that proceed to form either the 3'-PG residue (C4'-chemistry) or 3'-phosphate-ended fragments (C5'-chemistry) [24]. The partitioning of C4'-chemistry in a double-stranded lesion to produce either an abasic site or a strand break may have implications for the toxicity of the lesion *in vivo*.

Deoxyribose damage resulting from "activated bleomycin" is perhaps

one of the best understood of all these oxidative DNA-damaging agents [12,19,33-35]. As shown in Figure 0.1, the mechanism of deoxyribose degradation following C4' hydrogen abstraction has been shown to occur by two distinct pathways. The mechanism leading to the release of an alkali-labile 4'-keto 1'-aldehyde abasic site **8** (pathway A) has been well established [33,35], whereas the pathway which leads to the formation of 3'-PG and base propenals (pathway B) still remains obscure [12,19]. This mechanism is believed to involve the formation of a 4-peroxy radical **2** which is subsequently reduced to **3**. This in turn undergoes a Creigee-type rearrangement that ultimately leads to the formation of 3'-phosphoglycolate **6** and base propenal **7** [19]. Similarly, these products have been characterized for other genotoxic agents and the proposed mechanisms of their formation have been based on this model.

Extensive research has been done to elucidate the mechanisms by which 3'-PG-ended lesions are formed *in vitro* [12,29,36,37]. Limited research has been done, however, to directly address the question of the chemical mechanism of oxidative deoxyribose sugar damage *in vivo*. Damage in cells may differ from that *in vitro* studies due to factors unique to the cellular environment that have not been fully considered. In order to understand the chemistry of 3'-PG formation *in vivo*, the unique environment of the DNA (*i.e.* the presence of thiols, histone proteins, chromatin structure) must be taken into account when designing experiments to address this problem.

Glutathione, the main endogenous cellular non-protein thiol [38], is known to protect against toxic radical species. In an *in vitro* experiment involving the γ -irradiation of a plasmid restriction fragment, I show that glutathione inhibits the formation of 3'-PG. Determining whether this protection involves prevention of deoxyribose oxidation or reduction of

deoxyribose radical intermediates, thereby inhibiting DNA fragmentation, is an important research question as it relates to understanding the biological responses to radical-mediated DNA damage. The goal of this thesis is to characterize the C4'-chemistry induced by γ -radiation, peroxyxynitrite and calicheamicin, and to determine the effect of several factors (buffer composition, the presence of glutathione, and pH) on the formation of 3'-phosphate and 3'-PG by these agents. The results of these studies provide a means to begin to clarify the conflicting chemistries of the variety of small molecules that oxidize the deoxyribose sugar, such as Fe(II)-EDTA,[16], peroxyxynitrite [39-41], bleomycin [19,42] and the enediynes [15].

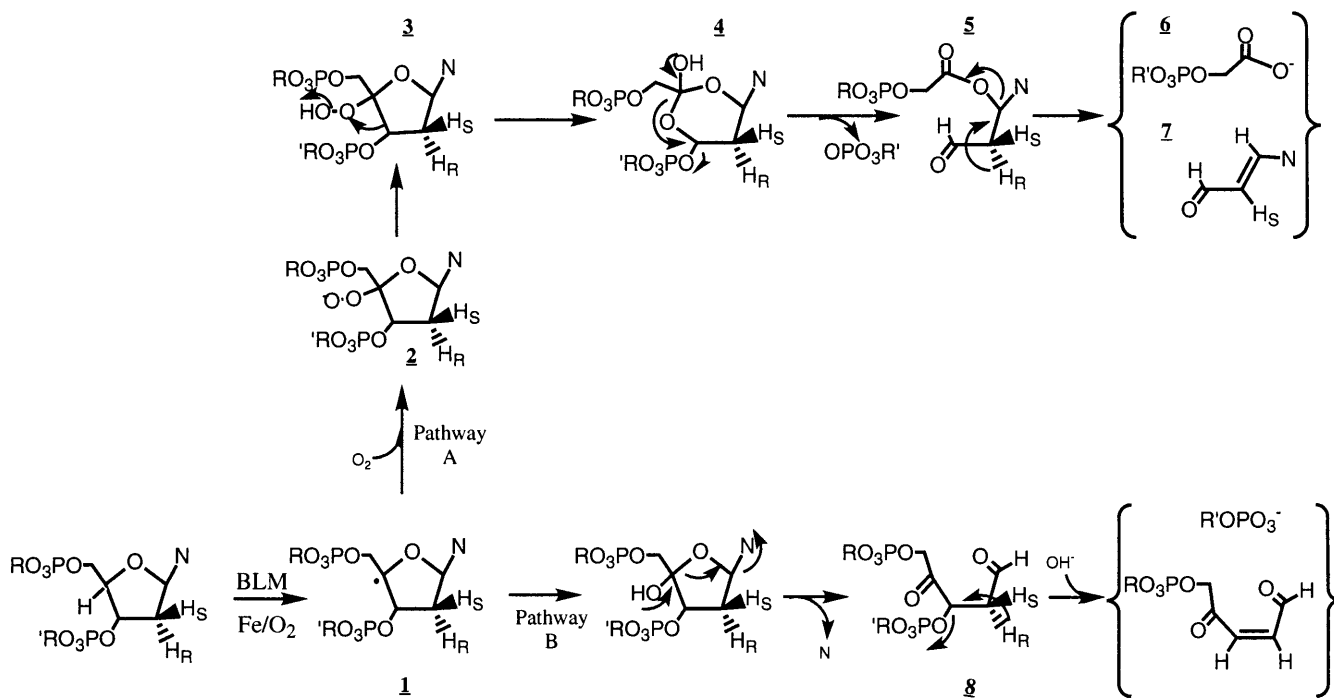


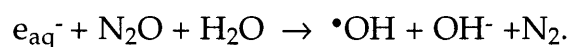
Figure 0.1. Bleomycin-induced C4'-chemistry. Proposed mechanism of BLM-mediated DNA damage.

Chapter I.
DNA Strand Breaks Produced by γ -Radiation and Peroxynitrite

Introduction

γ -Radiation Chemistry. The principle oxygen-centered radicals are: $\bullet\text{OH}$, $\text{O}\bullet^-$, $\text{O}_2\bullet^-$, $\text{HO}_2\bullet$, $\text{O}_3\bullet^-$, $\text{ROO}\bullet$, $\text{RO}\bullet$. Most of these species are implicated in the biological and toxic processes in cells [43,44]. The energy required to form free radicals from molecular compounds is provided by either high energy particles (e^- , p^+ , α) or by electromagnetic waves (γ -rays, synchrotron radiation, etc.) [29]. The high energy (typically MeV) of an incident particle or electromagnetic wave ultimately results in a large number of small energy deposits (~ 60 - 100 eV), each of which provides enough for ionization and excitation of an average of one or two molecules. In irradiated water or aqueous solutions, the resulting electrons, charged and neutral species, and non-radical species that are generated are e_{aq}^- , $\text{H}\bullet$, $\bullet\text{OH}$, H_2 , H_2O_2 [29]. The formation of these so-called primary species occurs in 10^{-10} seconds.

There are, however, a number of mechanisms by which these primary radicals can selectively be either scavenged or inter-converted [29]. The most important of the processes with respect to the oxygen radicals is the conversion of hydrated electrons into hydroxyl radicals as shown below:



Other significant reactions in an irradiated aqueous solution are the conversion of hydrated electrons into hydrogen atoms, the generation of superoxide and the removal of hydrogen atoms and hydroxyl radicals by alcohols. Finally, radiation chemistry provides the possibility of generating free radicals which in high polarity solvents are mainly homogeneously distributed and whose physical and chemical properties can selectively be studied.

von Sonntag and coworkers [23,29] have undertaken extensive studies of the DNA damage induced by γ -radiation, under conditions believed to

involve hydroxyl radical-mediated processes. Although most of the hydroxyl radicals add across the double bond of the nitrogenous bases, more than 20% of the hydroxyl radicals may directly abstract hydrogen atoms from deoxyribose [45]. In addition to the generation of abasic sites resulting from N-glycosylic bond labilization following base modification, numerous abasic sites have been shown to occur subsequent to hydrogen abstraction from the deoxyribose in γ -irradiated DNA. For example, Dizdaroglu *et. al.* [45] detected the formation of 2-deoxy-D-*erythro*-pentonic acid from calf thymus DNA irradiated under both aerobic and anaerobic conditions. This species is believed to arise by the abstraction of hydrogen from the C1'-position, followed by base release and the formation of the alkali-labile abasic site with a lactone at C1'. Evidence for this product has also been found in NCS-mediated strand scission [17,18].

Besides the formation of abasic sites subsequent to oxidative DNA damage, numerous deoxyribose degradation products are also known to occur [12,15]. Using γ -irradiation as a source of oxidative damage, the laboratory of Haseltine and co-workers identified two products resulting presumably from C4' hydrogen abstraction [27,28]. One of these products was a 3'-phosphoglycolate-ended DNA fragment which was differentiated from the other 3'-phosphate-ended fragments using high resolution gel electrophoresis. Further characterization of these products revealed that the more rapidly migrating of the two damage products contained the 3'-PG moiety. This lesion was suggested to contribute to the cytotoxic, mutagenic and carcinogenic effects of γ -irradiation based on a model in which insufficient repair of 3'-PG inhibits DNA polymerase [28]. However, Winters *et. al.* subsequently isolated three distinct enzymes from HeLa cells that were able to remove 3'-PG from both γ -irradiated and bleomycin-treated DNA [46]. These

enzymes all possessed class II AP endonuclease activity and were able to convert the lesions into substrates for DNA polymerases. Despite the isolation of these putative repair enzymes, the contribution of 3'-PG formation to the cytotoxicity associated with oxidative DNA damage *in vivo* still remains unclear.

The Chemistry of Peroxynitrite. The chemistry of the peroxynitrite (ONOO-) has been studied for decades [47]. A burst of interest in this inorganic peroxyacid occurred in 1990, however, when Beckman, Freeman and coworkers published a landmark paper which suggested that nitric oxide (NO) and superoxide (O₂⁻) could combine to form peroxynitrite *in vivo* [48]. The peroxynitrite anion is relatively stable, but its acid, peroxynitrous acid, rearranges to form nitrate with a half-life near 1 sec at pH 7 and 37 °C. The pK_A of HOONO has been determined to be 6.8 [49,50].

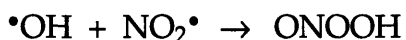
During its decomposition at physiological pH, peroxynitrite can produce some of the strongest oxidants known in a biological system, initiating reactions characteristic of the hydroxyl radical, nitronium ion and nitrogen dioxide [51]. Based on these observations, the toxic effect of peroxynitrite was postulated to be derived from the formation of hydroxyl radicals [52-54]. However, recent evidence suggests a more complicated picture and has led to a model in which an unstable, vibrationally-active peroxy-nitrous acid intermediate with the reactivity of hydroxyl radicals is involved in peroxynitrite-mediated damage [50].

By either mechanism, the genotoxic potential of peroxynitrite is likely to involve oxidation of both the bases and deoxyribose of DNA. As evidence of the latter, King *et. al.*, [39] have observed that peroxynitrite produces sequence-neutral strand breaks in DNA *in vitro*. Peroxynitrite was also shown by Kennedy *et. al.* [41] to produce the base damage product 8oxo-

guanine at levels higher to those observed for γ -radiation and Fe(II)-EDTA/H₂O₂. However, relatively little is known about the mechanism by which peroxynitrite initiates DNA cleavage or the identity of deoxyribose degradation products.

Koppenal *et. al.* have used empirical observations and thermodynamic hypotheses to suggest that peroxynitrite does not decompose homolytically into free hydroxyl radical and nitrogen dioxide [50]. The evidence that has been generated in support of a vibrationally active intermediate is as follows:

1) The rate constant for the reverse reaction,



has been estimated to be in the range of 10^{13} - 10^{15} M⁻¹ s⁻¹. This value exceeds the experimentally determined rate constant of 1.3×10^9 M⁻¹ s⁻¹ and the diffusion limited rate constant of 10^{10} - 10^{11} M⁻¹ s⁻¹.

2) The entropy of activation is small, (3 ± 2 cal/mole · K) compared to activation energies previously calculated for homolysis of RO-OR bonds which are generally on the order of ~ 12 cal/mole · K.

3) The hydroxyl radical scavengers DMSO and tert-butylalcohol do not affect the rate of peroxynitrite decomposition. If homolysis of HOONO does occur, it is expected that such potent hydroxyl radical scavengers would effect the rate of nitrate formation.

The ubiquitous nitric oxide (NO) has been implicated as both a physiologically-important signaling molecule [55-57] and a potent toxin and mutagen [58,59]. Peroxynitrite, formed by the reaction of NO with superoxide [48,49], represents one of several possible mediators of the genotoxic properties of NO [50]. Activated macrophages produce high levels of both NO and superoxide ([60] and references therein). It is thus possible that the cytotoxic properties of activated macrophages and the increased risk of cancer

associated with chronic inflammation are, in part, related to the production of peroxynitrite.

The objective of this study is to identify the deoxyribose degradation products arising from treatment of DNA with peroxynitrite by comparison with the known chemistries of γ -radiation. There are two questions that highlight the importance of these studies. First, does peroxynitrite produce damage via preferential attack of one or both grooves of DNA? Second, if peroxynitrite-mediated damage does not involve a hydroxyl radical intermediate, will the deoxyribose fragmentation products differ from those formed by other agents that produce hydroxyl radical such as γ -radiation and Fe(II)-EDTA/H₂O₂? Identification of the chemistry of the damage products in peroxynitrite-mediated DNA damage will provide answers to both of these questions. The effects of GSH and radical-scavenging buffers on the partitioning of C4'-chemistry will also be investigated.

Material and Methods

Chemicals. ONOOK/ KNO_3 was kindly provided by Dr. William A. Pryor, Biodynamics Institute, Louisiana State University. The plasmid pUC19 and restriction enzymes *Hind*III and *Eco*RI were purchased from New England Biolabs. *Pvu*II was obtained from Gibco-BRL. Calf intestine alkaline phosphatase and G-50 sephadex columns were obtained from Boehringer Mannheim. Putrescine dihydrochloride, hydrazine, phenol, chloroform and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were obtained from Sigma Chemical Co. Acrylamide/bisacrylamide (19:1) solutions and isoamyl alcohol were purchased from American Bioanalytical.

Description of γ -Radiation Source. DNA solutions were exposed to γ -radiation in a Gammacell-220 (Atomic Energy of Canada Ltd.). The Gammacell-220 utilizes an annular cobalt-60 (^{60}Co) source permanently enclosed within a lead shield, a stainless steel cylindrical drawer (for placement of samples), and a drive mechanism which moves the drawer up or down along the center line of the source. ^{60}Co decays with a 5.271 year half-life to stable Nickel-60 (^{60}Ni). During this decay process, ^{60}Co emits two cascades gamma rays with energies of 1.1732 MeV and 1.3325 MeV, for a combined energy of 2.5057 MeV. The dose rate of the Gammacell-220 during these experiments was calculated to be 5.55 Gy/min.

Source of Peroxynitrite. The reagent used to oxidize the end-labeled restriction fragment was a solid form of peroxynitrite generated by photolysis of potassium nitrate (KNO_3) [39]. This damaging agent was synthesized by irradiating reagent grade crystalline KNO_3 at 254 nm for 2 hr. under nitrogen gas at a temperature of 42 °C. This γ -irradiation produced a yellow solid which contained 26 μmol of ONOOK per gram of solid.

5'-[³²P] end-labeling of *Eco*RI-or *Hind*III-digested pUC19. The plasmid was uniquely labeled with [³²P] at the 5' end of either the *Eco*RI or *Hind*III cleavage site by successive application of alkaline phosphate, T4 kinase and [γ -³²P]ATP (150 μ Ci) [61]. Radiolabeled DNA was separated from the unincorporated labeled nucleotide using a G50-sephadex column, phenol:chloroform extraction followed by ethanol precipitation. The plasmid DNA was then digested with either *Hind*III or *Pvu*II to generate either a 50 bp or 143 bp restriction fragment, respectively. The radiolabeled DNA fragments of interest were subsequently separated from the larger fragments on a non-denaturing 12% polyacrylamide gel. The fragments of interest (see Figure 1.1 for sequence of *Eco*RI/*Hind*III pUC19 fragment and Figure 1.2 for sequence of *Hind*III/*Pvu*II pUC19 fragment) were then excised from the gel, eluted into gel elution buffer (300 mM sodium acetate, 1 mM EDTA pH 7.0), ethanol precipitated and resuspended in either deionized water or HEPES buffer (50 mM HEPES, 1 mM EDTA, pH 7.0).

3'-[³²P] end labeling of *Hind*III/*Pvu*II-digested pUC19. The supercoiled plasmid DNA was successively digested with *Hind*III and *Pvu*II and ethanol precipitated using standard procedures [61]. The DNA (10 μ g) was then resuspended in deionized water and reacted with [α -³²P]dideoxy ATP (50 μ Ci) and terminal deoxynucleotidyl transferase (100 units) in 100 mM sodium cacodylate, 2 mM CoCl₂, 200 μ M β -mercaptoethanol, pH 7.2, at 37 °C for 1 hr. The reaction was stopped by adding EDTA to a final concentration 5 μ M. The 3'-[³²P] labeled 143 bp fragment was then isolated from the larger fragment by resolving on a 12 % acrylamide gel and eluting as described earlier.

Preparation of Maxam-Gilbert chemical sequencing standards. Maxam-Gilbert chemical sequencing standards were prepared [62] and used in these experiments to serve as markers of either 3'- (for 5'- [³²P] labeled samples) or

5'- (for 3'-[³²P] labeled samples) phosphate-ended deoxyribose fragmentation products [24,30]. The 5'- and 3'-end-labeled 143 bp *HindIII/PvuII* fragments (7.2×10^5 cpm) were reacted separately in the presence of 30 $\mu\text{g}/\text{mL}$ sonicated calf thymus DNA (CT DNA) with either 1% (v/v) formic acid (purine-specific reaction) or 25 mM hydrazine (pyrimidine-specific reaction). Following these reactions, the DNA was cleaved at the damage sites by incubating at 90 °C with 100 mM piperidine. The samples were then lyophilized and resuspended in deionized water three times and finally resuspended in sequencing gel loading buffer (0.05% bromophenol blue, 0.05% xylene cyannol, 20 mM EDTA in 95% (v/v) deionized formamide).

γ -irradiation of 5'-[³²P] labeled *EcoRI/HindIII* pUC19. The chamber dose rate for the Gammacell-200 as of 2/1/93 was determined to be 6.4 Gy/min. Prior to each use the dose rate was calculated using an equation which relates the decay constant to the initial dose rate. Based on the dose rate at the time of irradiation, the radiolabeled restriction fragment (3.6×10^5 cpm/reaction) containing 30 $\mu\text{g}/\text{mL}$ CT DNA (unlabeled carrier DNA) in either deionized H₂O or 10 mM HEPES, 1 mM EDTA, pH 7.0, and controls were then γ -irradiated for varying lengths of time. The samples were kept in the cylindrical drawer of the Gammacell-200 for exposures of γ -radiation ranging between 4 and 1200 Gy. After irradiation, each sample was immediately placed on ice for at least 1 hr. prior to ethanol precipitation to ensure that each reaction had gone to completion.

Peroxynitrite Treatment of 5'- and 3'-[³²P] labeled *HindIII/PvuII* pUC19. DNA cleavage was initiated using a range of concentrations of peroxynitrite. Initially, the solid peroxynitrite was weighed in 1.5 mL Eppendorf tubes after which solutions of end-labeled DNA (3.6×10^5 cpm) containing 30 $\mu\text{g}/\text{mL}$ calf thymus DNA in 50 mM sodium phosphate and 0.1 mM diethylenetriamine-

pentaacetic acid (DETAPAC), pH 7.4 were added to the solid, vortexed for 30 seconds to initiate the reaction. The final concentrations of ONOO were 132, 264 and 528 μ M. The reactions were allowed to proceed at room temperature for 1 hr prior to desalting over G-50 sephadex quick spin columns and ethanol precipitation in preparation for chemical modification of the damage products.

The purified 5'-end labeled DNA was then split into thirds. The portions were either kept on ice for 1 hr as a control, treated with 100 mM putrescine dihydrochloride for 1 hr. at 37 °C to cleave all drug induced abasic sites to phosphate-ended fragments, or treated with 100 mM hydrazine (pH 7) for 1 hr at ambient temperature to convert the 4'-keto 1'-aldehyde abasic site to 3'-phosphopyridazine-ended strand breaks. A description of the various chemical treatments, the structures of possible products and their migrations relative to 3'-phosphate ended Maxam-Gilbert standards is described in Figure 1.3. Following these chemical modifications, the samples were again ethanol precipitated in preparation for gel resolution of the damage fragments.

Sequencing gel and PhosphorImager analysis. The C4'- and C5'-deoxyribose fragmentation products induced by treatment of the DNA with γ -radiation and ONOO- were identified by resolving the treated samples on 30 cm x 40 cm 20% polyacrylamide gels containing 8.3 M Urea. The identity of the sugar fragmentation products is determined by resolving the treated samples along with Maxam-Gilbert sequencing standards and making comparisons of the products based on the expected gel mobilities as described in Figure 1.3. Prior to loading on gels, 2.5×10^4 cpm aliquots of the samples were lyophilized, resuspended in 3 μ L sequencing gel loading buffer, boiled for 3 min, and chilled on ice for 3 min. Electrophoresis of the samples was then performed at 60 watts for 11-15 hr. The gels were then fixed in

sequencing gel fixing solution (10% acetic acid, 10% methanol), dried under vacuum and exposed to a phosphor screen for a minimum of 16 hours. The gel was scanned and analyzed using a Molecular Dynamics Phosphorimager and ImageQuant software.

Results

γ -irradiation of *EcoRI/HinIII* pUC19 in deionized H₂O. The purpose of this experiment was to serve as a positive control for the formation of 3'-PG, since this experiment has been previously done by Haseltine and coworkers [27,28]. Ultimately, the DNA damage products observed following γ -irradiation of this 5'-[³²P] labeled substrate will be compared to those produced subsequent to treatment with peroxyxynitrite. In this experiment the end-labeled fragment, containing 30 μ g/mL CT DNA in deionized H₂O, was exposed to 0, 4, 8, 16, 25, 50 and 100 Gy of γ -radiation. As shown in Figure 1.4, a linear dose response is observed as the exposure to γ -radiation is increased. A band migrating as expected for 3'-PG is also observed and shown to increase linearly with exposure to γ -radiation. This putative 3'-PG residue is consistent with the chemistries previously associated with C4'-hydrogen abstraction with γ -radiation, BLM, NCS and CAL γ_1^I .

Increased levels of γ -radiation are required to form 3'-PG when samples are irradiated in HEPES buffer. It was found that in the presence of 10 mM HEPES buffer, a substantial reduction in both overall DNA damaged and in 3'-PG was observed. Once, again, there is a linear dose response to increased exposure to γ -radiation and both 3'-phosphate- and 3'-PG-ended fragments were formed. As shown in Figure 1.5, a control for the levels of damage formed subsequent to a 16 Gy irradiation in deionized H₂O was also run on this gel so that direct comparison in the damage levels could be made. Evaluation of the gel reveals that nearly a 10-fold increase in exposure to γ -radiation was required to achieve the level of 3'-PG previously formed in deionized H₂O.

Glutathione inhibits the γ -radiation-mediated production of 3'-PG. Following the observed effect of HEPES on γ -radiation-mediated C4'-

chemistry, questions regarding the effects of physiologically relevant cellular components on the damage arose. Glutathione has been shown to offer protection against the destructive effects of reactive oxygen intermediates and free radicals in cells [38,63]. Therefore, using the sequencing gel analysis previously employed for γ -radiation in H₂O and HEPES buffer, the effect of GSH on the formation of 3'-PG was assayed. As shown in Figure 1.6, the relative amounts of 3'-PG formed as compared to the number of phosphate ended fragments is lowered in the presence of 50 mM GSH. This is consistent with a model in which GSH causes a shift in the C4'-chemistry by reducing the putative radical intermediates that lead to the formation of 3'-PG as proposed in Figure 1.7.

Evidence for the formation of 3'-PG in peroxynitrite-mediated damage. Putative 3'-PG-ended DNA fragments were observed when the 5'-end labeled fragment was treated with peroxynitrite as shown in Figure 1.8. Moreover, subsequent treatment with hydrazine led to the formation of a putative 3'-phosphopyridazine residue, which provides evidence for the presence of the 4'-keto 1'-aldehyde abasic site. Evidence for the formation of the 3'-glycol-aldehyde was sought by treating the ONOO- damaged DNA with sodium borohydride (see Figure 1.9). However, the results of this experiment were inconclusive.

An increase in the intensity of the 3'-phosphate-ended fragment, following cleavage with putrescine provides additional support for the existence of the abasic site [20]. The presence of the putative 3'-PG band in the ONOO-mediated damage is an important observation, because it serves as yet another example of an oxidizing agent that forms this product *in vitro*. Given the diverse group of oxidants that have been shown to produce 3'-PG, questions regarding the potential significance of this product arise, especially

as it relates to the cytotoxicity of these oxidizing agents.

Analysis of the C5'-chemistry induced by peroxynitrite. The *HindIII/PvuII* pUC19 fragment was 3'-ended labeled with [³²P] and treated with 528 μM peroxynitrite (samples contained 30 μg/mL CT DNA in 50 mM phosphate buffer pH 7.4) as described in Materials and Methods. The treated sample and control were then split in half and one portion was treated with 100 mM sodium borohydride to reduce the 5'-nucleoside aldehyde, which results from initial C5'-hydrogen abstraction, to the slower-migrating nucleoside. Although most of the damage appears to have 5'-phosphate ends (see Figure 1.10), there are several low intensity bands that may be the 5'-nucleoside aldehyde. The existence of this residue will have to be confirmed using other techniques such as HPLC and mass spectrometry.

Discussion

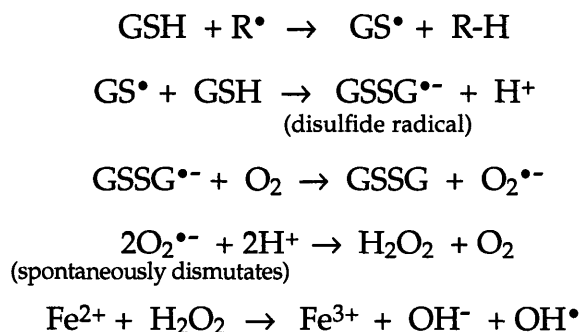
Under aerobic conditions, γ -irradiation of aqueous solutions produces several radical species that are able to cause strand breaks in DNA [29]. The radical-mediated damage is known to consist of lesions that result from direct oxidation of the nitrogenous base or the deoxyribose [45,64]. Here I focus on products that result from abstraction of the C4'-hydrogen atom from deoxyribose and report the effect of buffer composition and the presence of GSH on the nature of the products observed. In Figure 1.4, the formation of the γ -radiation-induced 3'-PG is shown and its formation is demonstrated to increase in a dose dependent manner.

The presence of radical scavengers is known to effect the amount of oxidative DNA damage produced with γ -radiation [29]. The purpose of comparing the levels of damage produced in deionized water to that in HEPES buffer was to identify factors which may either alter the production of 3'-PG. As shown in Figure 1.5, a nearly 10-fold increases in γ -radiation exposure is required to form 3'-PG in the presence of HEPES buffer. These results also revealed that the composition of the buffer can be modified in order to determine the effect of cellular components on the formation of 3'-PG. Through these *in vitro* experiments, new insights into factors which influence the formation of this 3'-PG *in vivo* can be obtained.

The environment of cellular DNA is believed to play a direct role in the chemistry of radical mediated damage. Numerous advances have been made in understanding the chemistry of oxidative DNA damage *in vitro*. However, the mechanisms of DNA damage *in vivo* have yet to be defined. In order to provide new insights into the role of specific cellular components on radical-mediated DNA damage, the effect of GSH on the formation of 3'-PG was investigated. GSH has been shown to offer protection against the

destructive effects of reactive oxygen intermediates and free radicals in cells [38,63]. As shown in Figure 1.6, GSH inhibits the formation of 3'-PG in an *in vitro* model involving γ -radiation of a plasmid restriction fragment (see 2000 Gy exposure in the presence of 50 mM GSH). However, it is unclear whether GSH serves primarily as a radical scavenger or reducer of the putative deoxyribose radical intermediates *in vivo*. Understanding the mechanism by which GSH offers protection against radical-mediated DNA damage may aid in the design of drugs to combat the deleterious effects of radical species on cells.

A possible role for GSH in the reduction of putative radical intermediates, which would explain the apparent shift from 3'-PG to 3'-phosphate-ended fragments, is based on a model presented by Ross *et. al.* [65]. Essentially, as GSH reduces these radical intermediates, it forms a glutathionyl radical (GS \cdot) which can lead to the formation of additional radicals *via* the reactions shown below:



In this model, the reduction of oxidized radical species by GSH leads to the formation of additional hydroxyl radicals (OH \cdot) which can lead to further oxidations of the DNA or chain termination events with existing deoxyribose sugar radicals. As shown in Figure 1.7, the presence of GSH in γ -irradiated solutions of DNA could shift the partitioning of the C4'-chemistry toward the formation of the 4'-keto-1'-aldehyde abasic site. This putative chain

termination reaction may explain the cellular protection provided by GSH against oxidative DNA damage *in vivo* [38,63].

Having investigated the role of GSH in γ -radiation-mediated damage, I was interested in characterizing the DNA damage products caused by ONOO⁻ and relating the chemistry to that observed with γ -radiation. Peroxynitrite (ONOO⁻), the product of product of superoxide reacting with nitric oxide, is a strong oxidant which is postulated to be long lived *in vivo* relative to hydroxyl radical [66]. The unusual stability of ONOO⁻ (relative to hydroxyl radical) may contribute to its toxicity by allowing it to react more selectively with cellular targets. A major factor contributing to peroxynitrite's toxicity may be its stability as an anion, even though ONOO⁻ is 36 kcal mol⁻¹ higher in energy than its isomer, nitrate [67].

King *et. al.*, demonstrated that ONOO⁻ produces non-specific deoxyribose fragmentation products typical of the hydroxyl radical [39], and Salgo *et. al.* and Kennedy *et. al.* [40,41] found that it causes nicks in plasmid DNA. Here I provide evidence for peroxynitrite-mediated formation of 3-phosphoglycolate, 4'-keto-1'-aldehyde and 5'-nucleoside aldehyde. These products have previously been observed in the oxidative DNA damage produced by γ -radiation [28,68], BLM [12,19,33], CAL γ_1^I [20,69], ESP A₁ [25,70] and NCS [14,24]. Once the products have been unambiguously characterized using analytical techniques such as HPLC/MS, the mechanism by which these products are formed and the nature of the oxidizing species may be determined. Obtaining knowledge about the structure of the "activated species" will further our understanding of this potent oxidant and could aid in designing agents to combat the toxic its effects of ONOO⁻ *in vivo*.

Based on the results of the gel mobility shift assay presented here, it is evident that ONOO⁻ produces 3'-PG following initial abstraction of the C4'-

hydrogen. These lesions may represent a genotoxic event, due to the observed requirement of repair enzymes to convert the damage to a substrate for DNA polymerase [46]. Moreover, the base propenal, a product shown to accompany the formation of 3'-PG in BLM [12,19] and γ -radiation damage, has been shown to be cytotoxic [71,72]. Evidence for the genotoxicity of the base propenal and other products of oxidative DNA damage is limited. However, Dedon *et. al.* recently found that the base propenal forms a mutagenic pyrimidopurinone adduct of guanosine following direct oxidation with BLM, CAL γ_1^I and ONOO [73]. These results suggest that, in addition to the direct genotoxicity of oxidative DNA lesions, the base propenal may also contribute to the mutagenic burden of a cell exposed to these agents. Furthermore, these results coupled with the formation of 3'-PG highlight the significance of C4'-chemistry as a mutagenic and genotoxic event *in vivo*.

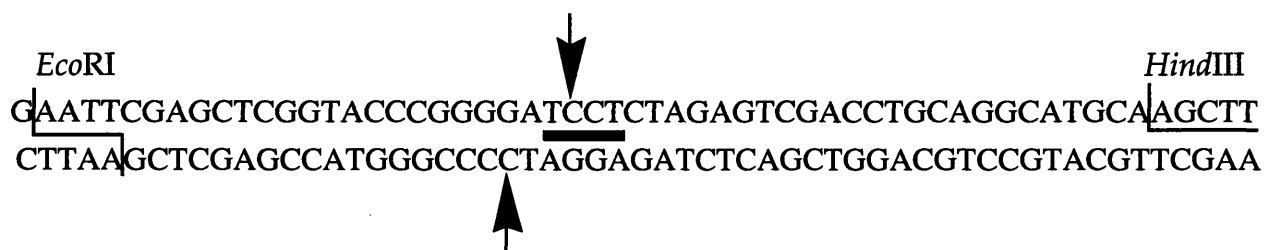


Figure 1.1. *EcoRI/HindIII* fragment of pUC19. Sites of CAL recognition (heavy bar) and damage sites (arrows) are shown.

5'

AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA
CCG AGC TCG AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT GAC TGG
GAA AAC CCT GGC GTT ACC CAA CTT AAT CGC CTT GCA GCA CAT
CCC CCT TTC GCC AG

3'

Figure 1.2. Sequence of 143 bp *HindIII* / *PvuII* pUC19 fragment. (single stranded) The CAL recognition sequence on the opposite strand (shown in Figure 1.1) is underlined.

Fragmentation Product	Chemical Modification	Observations on Gel
3' Phosphoglycolate	· No Treatment	Migrates ~ 1/4 bp faster than Maxam-Gilbert standards.
2' Deoxyribonolactone	· 100 mM Putresine	No new band, instead the intensity of phosphate band increases.
4' Keto 1' aldehyde abasic site	· 100 mM Hydrazine	New band appears with a slower migration than Maxam-Gilbert standards
3' Phosphoglyco-aldehyde	· 100 mM NaBH ₄	New band appears with a slightly faster migration than the aldehyde.

Figure 1.3. Expected products and gel shifts following chemical modification of oxidized 5'-[³²P] end-labeled DNA with putrescine, hydrazine and sodium borohydride.

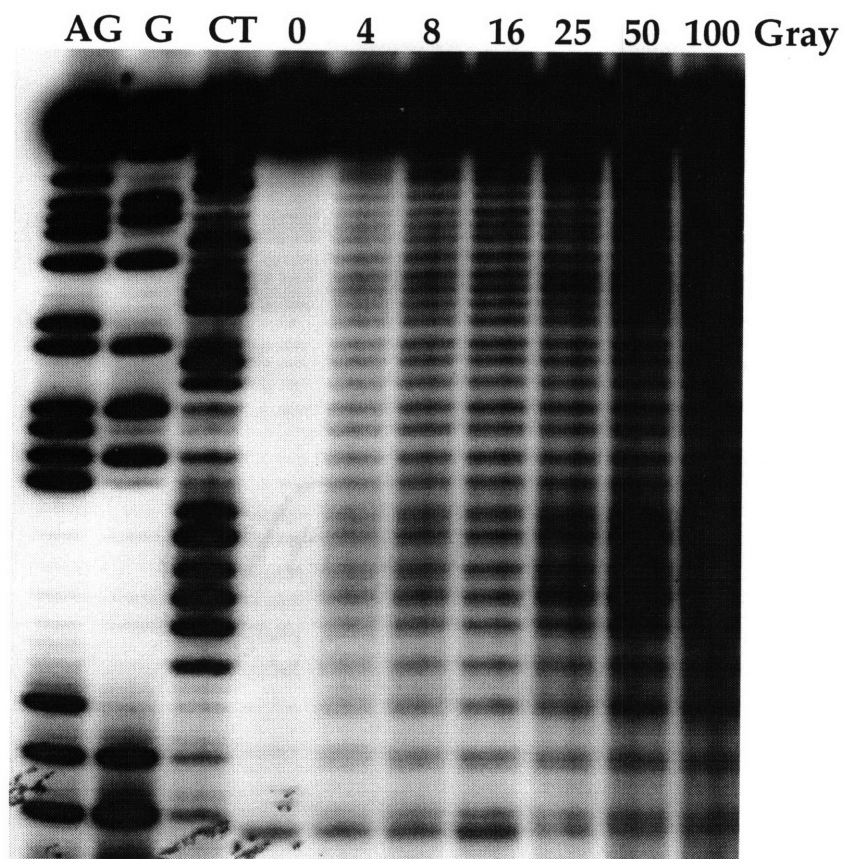


Figure 1.4. γ -irradiation of *EcoRI/HindIII* pUC19 fragment in deionized H₂O. The 5'-[³²P] end-labeled DNA was exposed to a 0-100 Gy range of γ -radiation to identify the 3'-phosphoglycolate ended damage product (band migrating $\sim 1/4$ of a base pair faster than the 3'-phosphate ended fragment). Lanes AG, G, and CT correspond to the Maxam-Gilbert sequencing standards.

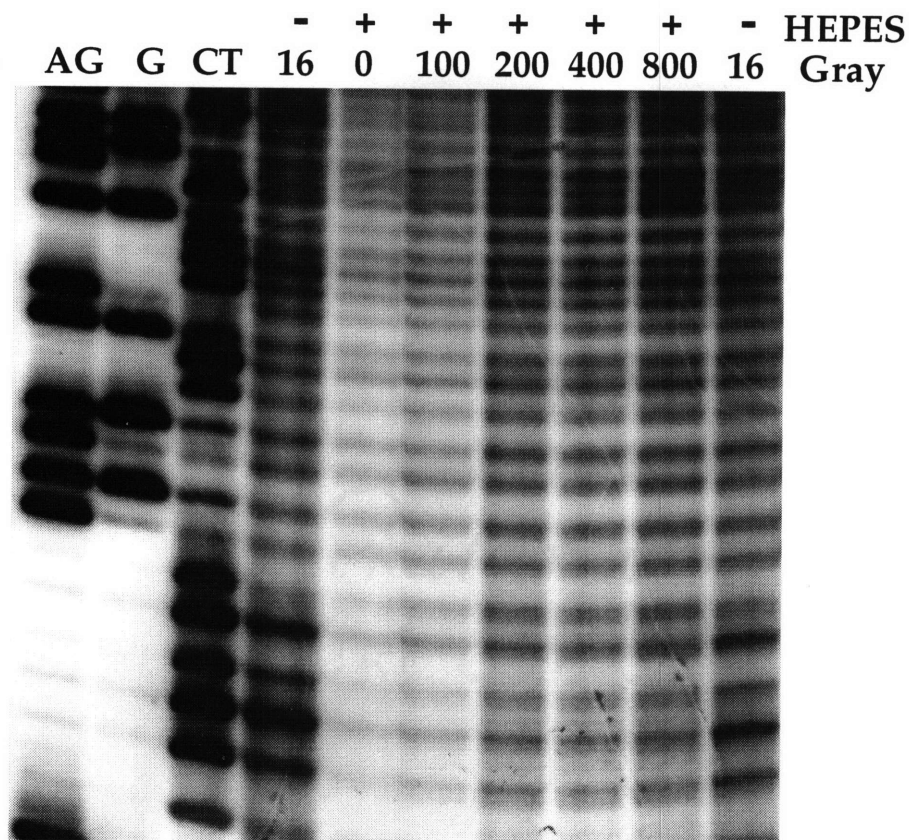


Figure 1.5. γ -irradiation of *EcoRI/HindIII* pUC19 fragment in HEPES buffer. The 5'-[^{32}P] end-labeled DNA was exposed to a 0-800 Gy range of γ -radiation in the presence of HEPES buffer (and 16 Gy in water) to determine the effects of the radical scavenging buffer on the overall levels of damage and the formation of the 3'-phosphoglycolate ended damage product. Lanes AG, G, and CT correspond to the Maxam-Gilbert sequencing standards.

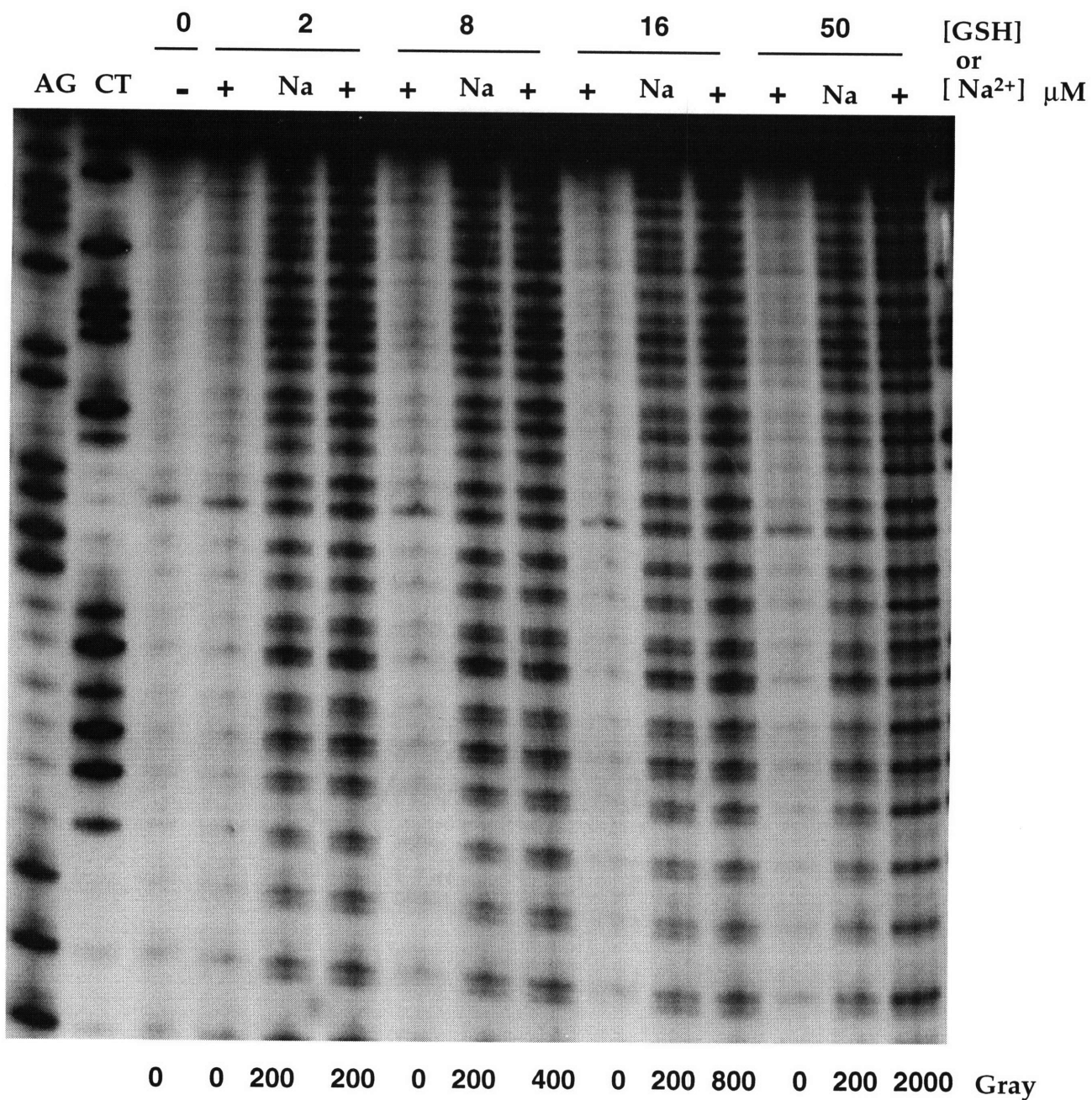


Figure 1.6. Effects of GSH and Na²⁺ on the formation of 3'-PG. In this experiment, the *EcoRI/Hind III* pUC19 fragment was γ -irradiated in the presence of increasing concentration of GSH and NaCl. The lanes containing NaCl are represented by the symbol(Na²⁺) above the gel. The plus (+) sign indicates samples which contained GSH. The exposures to γ -radiation are shown at the bottom of the gel. Lanes AG and CT correspond to Maxam-Gilbert sequencing standards.

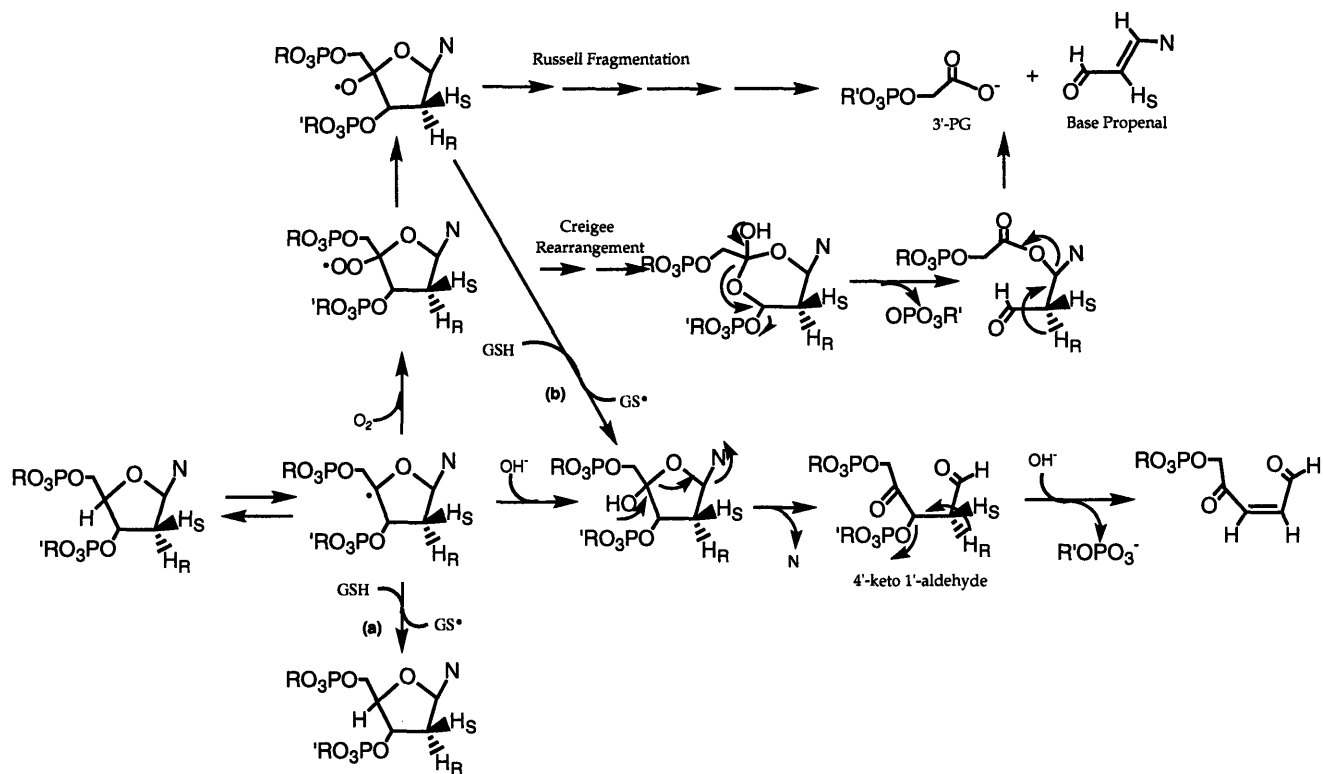


Figure 1.7. Proposed role of glutathione in protecting cells against radical-mediated DNA damage (a) and partitioning of C4'-chemistry toward the formation of the 4'-keto 1'-aldehyde abasic site as opposed to 3'-PG (b).

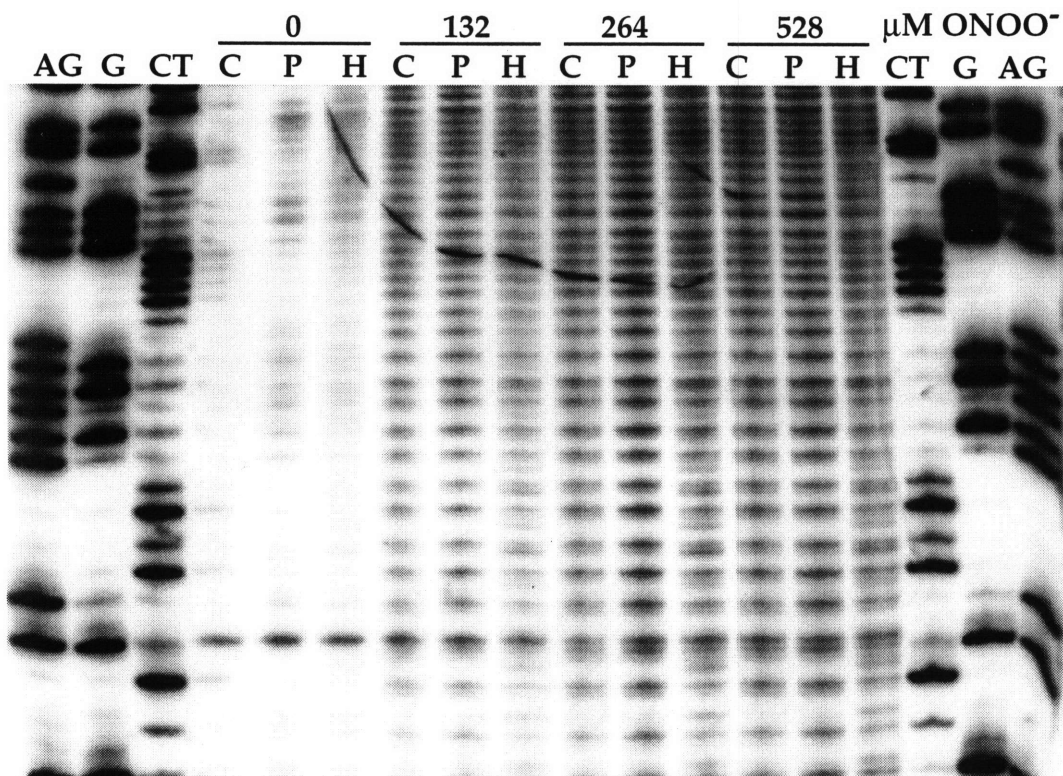


Figure 1.8. Evidence for the formation of ONOO-mediated 3'-phosphoglycolate. The 5'-[³²P] end-labeled *Hind*III/*Pvu*II pUC19 fragment was treated with 0,132, 264 and 528 μM ONOO. Reactions with Putrescine (P) indicates the presence of abasic sites (increased intensity of phosphate band in these lanes). Reactions with Hydrazine (H) provides evidence for the existence of 4'-keto-1'-aldehyde (based on the putative 3'-phosphopyridazine bands in these lanes). Lanes AG and CT represent Maxam-Gilbert chemical sequencing standards.

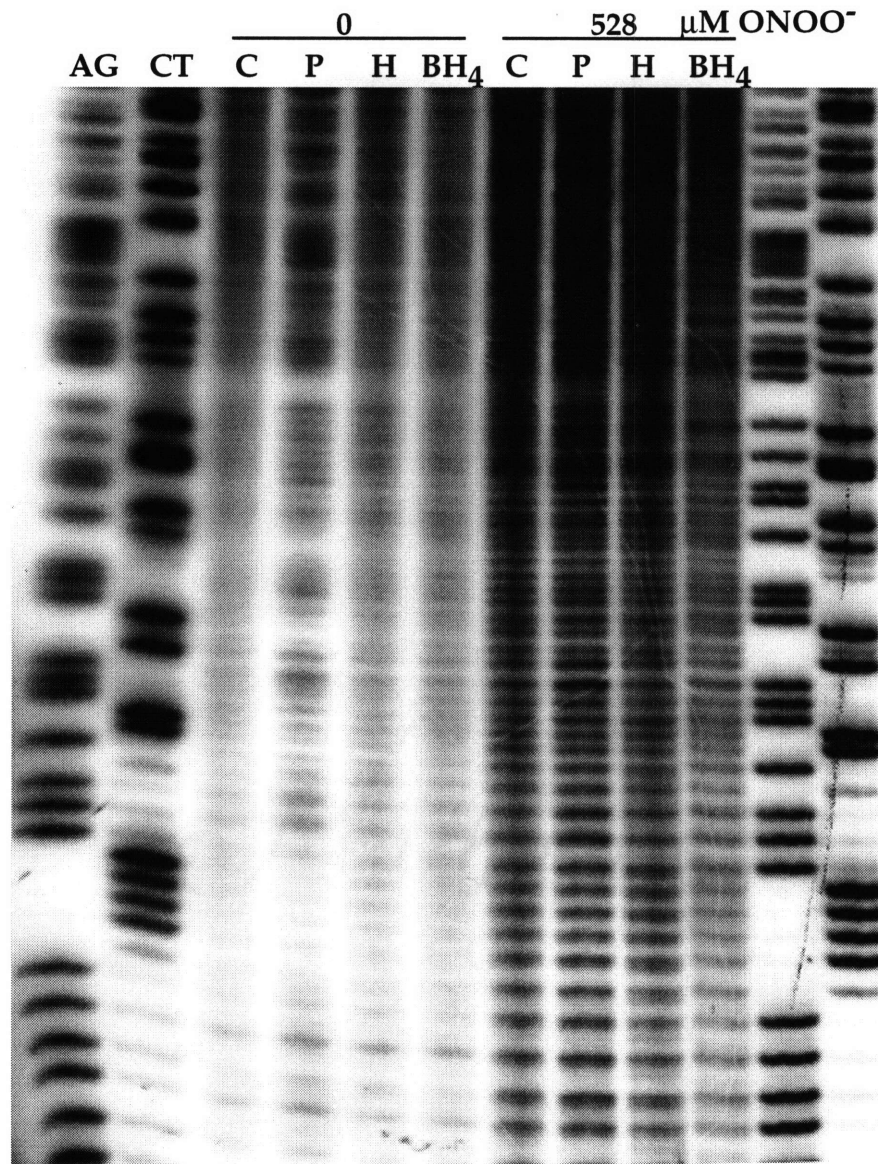


Figure 1.9. Putative formation of 3'-glycoaldehyde by peroxynitrite. Treatment of 5-[³²P] end-labeled *Hind*III/*Pvu*II pUC19 fragment with 528 μM ONOO⁻ and subsequent analysis of damage chemistry *via* modification of oxidized deoxyribose products with putrescine (P), hydrazine (H) and sodium borohydride (BH₄; reduces putative 3'-glycoaldehyde to slower migrating species). Lanes AG and CT represent Maxam-Gilbert chemical sequencing standards.

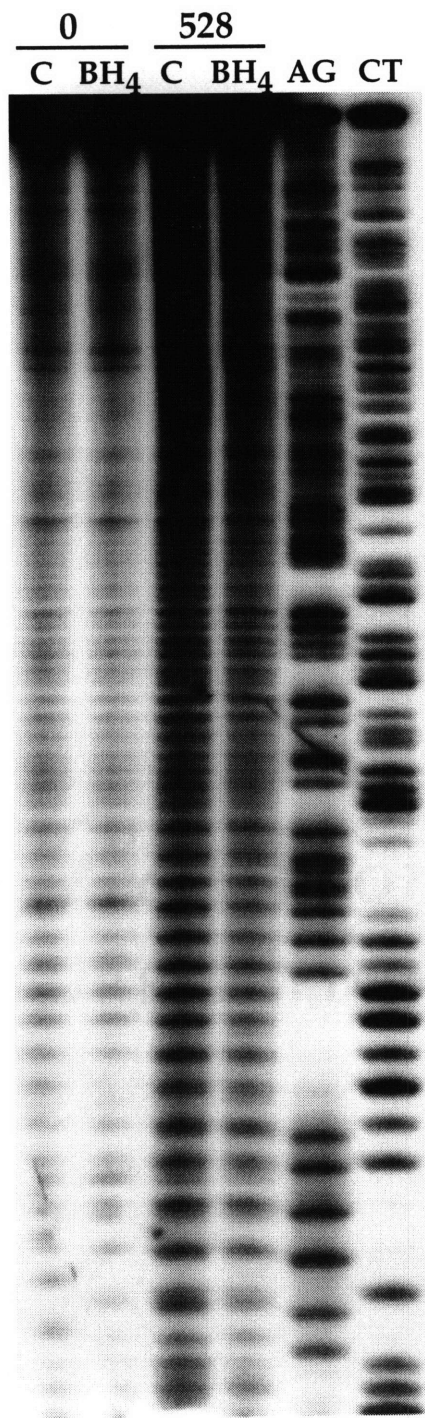


Figure 1.10. Analysis of C5'-chemistry induced by peroxynitrite. The 3'-[³²P] end-labeled *Hind*III/*Pvu*II pUC19 fragment was reacted with 0 and 528 μM ONOO⁻ and subsequently treated with sodium borohydride (BH₄) to reduce the putative 5'-nucleoside aldehyde to the slower migrating nucleoside residue.

Chapter II.
C4'-Chemistries-Induced by Calicheamicins γ_1^I and θ_1^I

Introduction

Calicheamicin γ_1^I (CAL γ_1^I) [74,75], a member of the enediyne family of anti-tumor antibiotics (see figure 2.1 for structure) isolated from fermentations of *Micromonospora echinospora ssp. Calichensis*, has been shown to interact with double-helical DNA causing site-specific double-stranded cleavage [20,76]. The observed cleavage occurs predominantly at TCCT, TTTT and CTCT sequences [76] and results from the unique positioning of the drug in the minor groove of DNA [77,78]. However, CAL γ_1^I appears to interact with DNA by recognition of local DNA conformation or flexibility [79,80] rather than a direct reading of these DNA sequences. This conclusion is based in part on the number of different sequences damaged by CAL γ_1^I [79,81,82] and the dependence of the damage frequency on the flanking sequences [81] or the intrinsic flexibility of the recognition site [80].

CAL γ_1^I contains several unusual structural features, including a glycosylated hydroxylamino sugar, an enediyne moiety and a labile methyltrisulfide group. Reduction of the methyltrisulfide by thiols leads to a Bergmann-type rearrangement of the enediyne moiety to a nondiffusible 1,4-dehydrobenzene diradical species which initiates oxidative strand scission by hydrogen abstraction from deoxyribose [15,83]. The resulting sugar radicals then proceed to interact with molecular oxygen to undergo position-specific degradation reactions resulting in abasic sites or strand breaks.

Single molecules of CAL γ_1^I have been shown to produce predominantly double-stranded lesions [20], with the typical three base pair staggering of the damage in a 3'-direction indicative of minor groove binding [15,81,84]. Moreover, isotope transfer studies, hydroxyl radical footprinting and the NMR solution structure of the CAL γ_1^I /DNA complex all suggest that the drug binds to target sequences with its carbohydrate tail extending toward the

5'-end of the purine strand of its recognition sequences [69,76-78,85]. The tail, which appears to adopt a right-handed screw which complements that of the minor groove [86], has been demonstrated to be responsible for the sequence-selectivity of the drug and is a major contributor to its binding energetics [86-88].

Calicheamicin θ_1^I (CAL θ_1^I), (see Figure 2.1 for structure) a synthetic analogue of CAL γ_1^I , was designed to differ only with respect to its triggering device [89]. Whereas CAL γ_1^I has a methyltrisulfide trigger which is activated by thiols such as GSH, CAL θ_1^I has a thioacetyl group that undergoes hydrolysis under mildly basic conditions. Once activated, CAL θ_1^I has been shown to yield high levels of double-stranded breaks with the same sequence selectivity and damage chemistry observed with CAL γ_1^I as I demonstrate here. The recognition and cleavage of TCCT, TTTT, and CTCT sites of helical DNA by CAL θ_1^I is also attributed to its oligosaccharide tail which is identical to that of CAL γ_1^I . As previously discussed, the carbohydrate tail has been shown to contribute to the selective binding to these sequences in the parent compound [90]. Moreover, the potency of CAL θ_1^I as a DNA cleaving agent in neutral or basic media was shown by Nicolaou *et. al.* to be further potentiated by thiols as expected from the higher nucleophilicity of sulfur nucleophiles as compared to oxygen-centered nucleophiles.

Surprisingly, there was an increased cytotoxicity associated with CAL θ_1^I compared to CAL γ_1^I (up to 1000-fold) in certain cell lines [89]. There are several possible hypotheses which may account for this phenomenon: (1) CAL θ_1^I is more efficient at producing DNA damage than CAL γ_1^I in these cell lines; (2) CAL θ_1^I targets different regions of the genome than CAL γ_1^I in the cell lines; (3) CAL θ_1^I targets other as yet undetermined cellular targets (*i.e.* RNA, proteins, lipids, or carbohydrates). However, based on the

observation that nearly 10^3 molecules of the drug is needed to kill a cell, (Xu, unpublished results) it is unlikely that any species of RNA, protein, lipid or carbohydrate can be the predominant cytotoxic cellular target. Likewise, CAL θ_1^I would have to show greater selectivity for critical gene loci than CAL γ_1^I for the second hypothesis to be true. Thus, it more likely that the different triggering moieties contribute greatly to the different potencies in producing DNA damage at the genomic level, thereby making CAL θ_1^I a more efficient cell killing molecule than CAL γ_1^I . Ongoing research in our laboratory designed to test these hypotheses is currently underway.

As shown here, CAL θ_1^I and CAL γ_1^I produce damage at the same sequences. However, the identity of the deoxyribose fragmentation products formed by these drugs has not been clearly defined. The goal of this research is to compare the deoxyribose fragmentation products formed by CAL θ_1^I to those previously characterized for CAL γ_1^I . The unique activation of CAL θ_1^I , which unlike CAL γ_1^I does not require thiols, provides an opportunity to determine the effect thiols on product formation. Furthermore, through comparisons of the C4'-chemistries of CAL θ_1^I versus CAL γ_1^I new insights into the effects of thiols on the partitioning of the damage between the 4'-keto-1'-aldehyde abasic site and strand breaks consisting of 3'-PG and 5'-phosphate ends will be obtained.

Material and Methods

Chemicals. Calicheamicin γ_1^I was kindly provided by Dr. G. Ellestad, Wyeth-Ayerst Research; calicheamicin θ_1^I was provided by Dr. K. C. Nicolaou, Scripps Research Institute. Restriction enzymes *Hind*III and *Pvu*II in addition to pUC19 were purchased from New England Biolabs and Gibco-BRL, respectively. Calf intestine alkaline phosphatase and G-25/G-50 sephadex columns were obtained from Boehringer Mannheim. Complementary oligonucleotides (20-mer) CAL3 and CAL4 were synthesized by Genosys. Putrescine dihydrochloride, hydrazine, GSH, γ -glutamyl-glycine (Glu-Gly) and TEMED were obtained from Sigma. Acrylamide/bisacrylamide (19:1) solutions were purchased from American Bioanalytical.

5'- [32 P] end-labeling and annealing of oligonucleotides. Aliquots (3 μ g) of the single-stranded 20-mer CAL4 (see Figure 2.2 for structure), were labeled with [32 P] at the 5'-end by incubation with 150 μ Ci [γ - 32 P]ATP and 10 units T4 kinase for 1 hr. at 37° C (61). The reaction was stopped by addition of EDTA (final concentration of 50 μ M) and removal of unincorporated nucleotides on a G-25 sephadex column. Finally, a 3-fold excess of the complementary oligonucleotide CAL3 (see Figure 2.2 for structure) was added to the 5'-end labeled DNA, boiled for 3 min, and slowly cooled to room temperature for several hours. The double-stranded oligonucleotide was ethanol precipitated and resuspended in either 50 mM sodium phosphate, pH 7.4 or HEPES buffer (50 mM HEPES, 1 mM EDTA, pH 7.0).

5' -[32 P] end-labeling of *Hind*III digested pUC19. The plasmid was uniquely labeled with [32 P] at the 5'-end of the *Hind*III cleavage site by successive application of alkaline phosphates, T4 kinase and [γ - 32 P]ATP [61]. The plasmid DNA was then run over a G-50 sephadex column and digested with *Pvu*II to generate a 143 bp fragment, which was subsequently purified

from the larger 185 bp fragment on a non-denaturing 12% polyacrylamide gel. The fragment of interest was then excised from the gel and the DNA was eluted by the crush-and-soak method. DNA was then purified by ethanol precipitation and resuspended in HEPES buffer.

Preparation of Maxam-Gilbert chemical sequencing standards. Maxam-Gilbert chemical sequencing standards of the 143 bp *HindIII/PvuII* fragment and 20-mer oligonucleotide were prepared and used in these experiments to serve as markers of 3'-phosphate ended deoxyribose fragmentation products [62]. The 5'-end labeled DNA substrates ($\sim 5 \times 10^5$ cpm) were reacted in the presence of 30 $\mu\text{g}/\text{mL}$ sonicated calf thymus DNA (CT DNA) with either 1.0% (v/v) formic acid (purine-specific reaction) or 25 mM Hydrazine (pyrimidine specific-reaction). Following these reactions, the DNA was cleaved at the damage sites by incubating at 90 °C with 100 mM piperidine. The samples were then lyophilized and resuspended in double deionized water thrice and finally resuspended in sequencing gel loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol 20 mM EDTA in 95% (v/v) deionized formamide).

Treatment of 20-mer with CAL γ_1^I and CAL θ_1^I . Initially, DNA stock solutions consisting of 5'-[^{32}P] end-labeled 20-mer (CAL4), 30 $\mu\text{g}/\text{mL}$ CT DNA and 5 mM GSH or 5 mM Glu-Gly in either phosphate buffer or HEPES buffer. The reactions were initiated by adding (2-6 μL) aliquots of CAL γ_1^I and CAL θ_1^I dilutions to portions of the DNA stock (final volume = 100 μL) and reacting at 37 °C for 1 hr. After the incubation, the reaction mixture containing the damaged 5'-end labeled oligonucleotide was split into thirds. The portions of the CAL γ_1^I and CAL θ_1^I treated samples were either kept on ice for 1 hr. as a control, treated with 100 mM putrescine dihydrochloride for 1 hr. at 37 °C to cleave all drug induced abasic sites to phosphate-ended fragments, or treated with 100 mM hydrazine (pH 7) for 1 hr. at ambient temperature to convert the

4'-keto 1'-aldehyde abasic site to 3'-phosphopyridazine-ended strand breaks.

Treatment of *HindIII/PvuII* fragment with CAL γ_1^I and CAL θ_1^I . DNA damage reactions of the end-labeled *HindIII/PvuII* fragment were initiated by adding aliquots (2-6 μ L) of calicheamicin stock solutions in methanol to mixtures containing 30 μ g/mL CT DNA and 5 mM GSH and/or 5 mM Glu-Gly in HEPES buffer (final volume = 100 μ L). The reactions were allowed to proceed for 1 hr. at 37 °C. Following treatments with CAL γ_1^I and CAL θ_1^I , the 5'-[32 P] end-labeled fragment was reacted with 100 mM putrescine and 100 mM hydrazine as described for the 20-mer.

Sequencing gel and phosphorimager analysis. The CAL γ_1^I and CAL θ_1^I treated samples were ethanol precipitated to desalt samples in preparation for resolution of damage chemistries on either 30 cm x 40 cm (20-mer) or 30 cm x 60 cm (143 bp fragment) 20% polyacrylamide gels containing 8.3 M Urea. Prior to loading on gels, 2.5×10^4 cpm aliquots of the samples were lyophilized, resuspended in 3 μ L sequencing gel loading buffer, boiled for 3 min, and chilled on ice for 3 min. Electrophoresis of the samples was then performed at 60 watts for 12-15 hr. (30x40 cm gels) or at 75 watts for 18-20 hr. (in 30x60 cm gels). The gels were then fixed in sequencing gel fixing solution (10% acetic acid, 10% methanol), dried under vacuum and exposed to a phosphor screen for a minimum of 16 hr. The image was scanned using a Phosphorimager (Molecular Dynamics) and analysis was performed using ImageQuant software.

Results

Effect of buffer on the distribution of CAL γ_1^I -mediated C4'-chemistry. The annealed 5'-[^{32}P] end-labeled 20-mer (CAL34) was treated with CAL γ_1^I (1 mM) in the presence of HEPES buffer (pH 7.5) or 50 mM phosphate buffer (pH 7.5) at 37 °C for 1 hr. The purpose of this experiment was to determine the effect of these two buffers on the distribution of the C4'-chemistry induced by the drug. There was increased damage at the recognition sequence in the presence of HEPES buffer compared to phosphate buffer (see Figure 2.3). However, the level of damage appears to be greater in the secondary recognition sequence (data not shown). The resolution of 3'-PG from the 3'-phosphate ended fragment was not complete in this experiment, but subsequent PhosphorImager analysis of the gel revealed that there was an identical relative formation of the product in the presence of either HEPES or phosphate buffers. Moreover, the appearance of the expected 3'-phosphopyridazine band following treatment with hydrazine confirms the existence of C4'-chemistry in the presence of both buffers.

Different relative levels of 3'-phosphoglycolate and 3'-phosphate ended fragments are produced by CAL γ_1^I and CAL θ_1^I . The 5'-[^{32}P] end-labeled 20-mer was used in these experiments and treated with a range of CAL γ_1^I concentrations (0-1 μM) and 10 μM CAL θ_1^I in the presence of 10 mM GSH and 10 mM Glu-Gly, respectively. The pH of the GSH and Glu-Gly solutions were determined to be ~7.5. The samples also contained 30 $\mu\text{g}/\text{mL}$ CT DNA and were incubated at 37 °C for 1 hr. (CAL γ_1^I treatment) and 48 hr (CAL θ_1^I treatment) in 50 mM phosphate buffer, pH 8.5 . As seen in Figure 2.4, a dose response was observed over the 0-1 μM range of CAL γ_1^I and the amount of CAL θ_1^I required to cause the same level of damage as the 1 μM CAL γ_1^I treatment was ~10-fold greater.

Whereas the ratio of 3'-PG/3'-phosphate appears to be ~1.0 in the CAL γ_1^I -treated samples, the ratio is > 4.0 in the CAL θ_1^I -damaged lanes. It is possible that this increased production of 3'-PG in CAL θ_1^I -mediated damage may be related to its increased cytotoxicity in certain cell lines [89]. Furthermore, there was no evidence for the 3'-phosphopyridazine fragment in either the CAL γ_1^I or CAL θ_1^I treatments (see Figure 2.4). This may be the result of hydrolysis of the 3'-phosphopyridazine or modification of the 4'-keto-1'-aldehyde abasic prior to treatment with hydrazine due to the fact that the pH of the buffer was raised to 8.5 in this experiment to ensure activation of CAL θ_1^I .

Increased damage is observed when longer sequences of DNA are treated with both CAL γ_1^I and CAL θ_1^I . Because of the high concentration of CAL θ_1^I and the long incubation times that were required to observe the damage using the CAL34 oligomer, I decided to use the 143 bp *HindIII/PvuII* pUC19 as a DNA substrate to compare the damage chemistries-induced by CAL γ_1^I and CAL θ_1^I . Preliminary results in our laboratory have suggested that CAL γ_1^I may find its recognition sequences by "tracking" along DNA sequences (Dedon, unpublished results). Furthermore, the length of the DNA sequence is postulated to have an effect on the amount of DNA that the drug produces. Comparisons of the levels of CAL γ_1^I and CAL θ_1^I -mediated damage observed with the 20-mer CAL34 and the 143 bp *HindIII/PvuII* fragment may provide evidence in support of this hypothesis.

The 5'-[^{32}P] end-labeled *HindIII/PvuII* fragment was treated with 0.4 μM CAL γ_1^I or 2 μM CAL θ_1^I in 50 mM HEPES, 1 mM EDTA, pH 7.0, containing 30 $\mu\text{g}/\text{mL}$ CT DNA and incubated at 37 °C for 1 hr. Following these treatments, the DNA was treated with putrescine and hydrazine as previously described. In this experiment, the resolution of the 3'-PG ended fragment

(from the 3'-phosphate ended fragment) in the CAL γ_1^I -treated lane was not complete (see Figure 2.5). Treatment of the CAL γ_1^I -damaged DNA with hydrazine led to the expected 3'-phosphopyridazine product which is indicative of the presence of the 4'-keto-1'- aldehyde abasic site.

Apparently there was no reaction in the CAL θ_1^I -treated lane which may be due to the neutral pH of the buffer. However, there was evidence for the formation of both 3'-phosphate and 3'-phosphopyridazine in the CAL θ_1^I -treated samples following reaction with hydrazine. This suggests that the drug was inactive during the initial 1 hr incubation and that the addition of hydrazine activated CAL θ_1^I . Additionally, the drug was able to produce damage products (at the same sequences as CAL γ_1^I) consisting of both 3'-phosphate (possibly 3'-PG) and 4'-keto-1'- aldehyde abasic sites.

Apparent activation of CAL θ_1^I by hydrazine. The experiment described above with the *HindIII/PvuII* was repeated with the following modifications in the experimental design. In order to determine the effects of GSH activation on the chemistries observed with CAL γ_1^I and CAL θ_1^I , three sets of reactions were performed. The three sets were treated with 0.4 μ M CAL γ_1^I and 1 μ M CAL θ_1^I in the presence of either: (1) 5 mM GSH, (2) 5 mM Glu-Gly or (3) 2.5 mM GSH/2.5 mM Glu-Gly.

5 mM GSH reactions: The primary recognition sequence (5'-TCCT-3') is damaged in the unmodified CAL γ_1^I treated lane (prior to putrescine and hydrazine treatments). The expected products indicative of C4'-hydrogen abstraction were observed after treatment with putrescine and hydrazine (see Figure 2.6). There was no damage in the unmodified CAL θ_1^I -treated samples. However, the putrescine- and hydrazine-treated samples had substantial damage at the primary recognition sequence.

5 mM Glu-Gly reactions: As expected, treatment with CAL γ_1^I resulted

in no damage in the primary sequence (or any secondary sequences) prior to or after reactions with putrescine and hydrazine. This result confirms that Glu-Gly is acting merely as an ionic strength control and does not play a role in activation of the drug. Also there was there was a substantial level of damage in the unmodified CAL θ_1^I -treated samples. Minimal damage was observed following the reaction with putrescine and following treatment with hydrazine there was evidence for the 4'-keto-1'-aldehyde (see Figure 2.6). This result suggests that both of these amines are able to activate CAL θ_1^I . Unlike CAL γ_1^I , which requires thiol activation, CAL θ_1^I is probably a more cytotoxic compound *in vivo* due to the multitude of compounds (including hydroxide ion) which can facilitate its activation.

2.5 mM GSH/2.5 mM Glu-Gly reaction: Following treatment with CAL γ_1^I , there is evidence for C4'-chemistry in the unmodified DNA and in the putrescine- and hydrazine-treated DNA. The presence of GSH (which activates the drug) is responsible for the appearance of the C4'-damage products (see Figure 2.6). There was no damage in the unmodified CAL θ_1^I -treated DNA and there was minimal damage in the putrescine-treated samples. However, there were substantially greater levels of damage in the hydrazine-treated samples. The fact that the buffer is at pH 7.0 is most likely responsible for the lack of damage in the unmodified CAL θ_1^I treatments.

Discussion

Calicheamicin γ_1^I , an enediyne anti-tumor antibiotic, has been studied extensively *in vitro* and has been shown to produce predominantly double-stranded breaks in duplex DNA upon incubation with thiols [20]. Recently, a structurally related analogue (CAL θ_1^I) was synthesized [89] which, unlike the naturally occurring compound (CAL γ_1^I), can be activated by hydrolysis in alkaline solutions. In this thesis, the deoxyribose fragmentation products resulting from C4'-hydrogen abstraction by these two compounds were characterized using gel mobility shift assays. In particular, comparisons of the levels of 3'-PG formed by these compounds were made in an effort to relate the formation of this lesion to the genotoxicity of these damaging agents. Additionally, the effects of factors such as buffer composition, pH, length of DNA, and the presence of GSH were addressed.

As shown in Figure 2.3, there was identical production of 3'-PG in both the HEPES and phosphate buffers. However, there was ~25% more total damage in the HEPES buffer. The relative formation of 3'-PG following treatments with putrescine and hydrazine could not be determined in this experiment, because the DNA was not purified prior to these reactions. Moreover, as demonstrated here, these amines are able to activate CAL θ_1^I and could alter levels of 3'-PG and 3'-phosphate produced by the drug.

Putrescine is known to express abasic sites as strand breaks with 3'-phosphate-ended fragments [20]. The results of this experiment suggest that either putrescine is much more efficient at expressing abasic sites as strand breaks in HEPES buffer, or that the levels of abasic site formation are greater in the presence of HEPES buffer. The observed increase in total damage in the presence of HEPES may provide support for the latter possibility. However, from this experiment there is no evidence that would distinguish between

these two possibilities.

Treatment of the 20-mer CAL34 with a range of CAL γ_1^I concentrations (0-1 μM) and 10 μM CAL θ_1^I in 50 mM phosphate buffer revealed that the levels of 3'-PG was substantially greater with CAL θ_1^I than with CAL γ_1^I (see Figure 2.4). Over the range of CAL γ_1^I concentrations used, the percent 3'-PG as a fraction of the total damage increased from ~30% to ~50%. However, the percent of 3'-PG in the 10 μM CAL θ_1^I -treatment was >80%. This apparent increased formation of 3'-PG in the CAL θ_1^I -treated samples may explain the increased cytotoxicity of CAL θ_1^I as compared to CAL γ_1^I in certain cell lines [89]. Comparisons with equal concentrations of CAL γ_1^I need to be made to determine whether or not the increased formation of 3'-PG observed in the CAL θ_1^I -treated samples is dose dependent. Moreover, the fact that the CAL θ_1^I -treated sample was incubated for 48 hr. (*versus* 1 hr for CAL γ_1^I -treated samples) may have contributed to the differences described above. Finally, reactions of both the CAL γ_1^I - and CAL θ_1^I -treated samples with hydrazine did not result in the formation of the expected 3'-phosphopyridazine which is indicative of the presence of the 4'-keto-1'-aldehyde abasic site. The fact that the pH was 8.5 in these reactions may have led to a hydrolysis of this abasic site and hence a reduction in the 3'-phosphopyridazine product.

Because of the high concentration of CAL θ_1^I and the long incubation times that were required to see the damage using the CAL34 oligomer, I decided to use the 143 bp *HindIII/PvuII* pUC19 as a DNA substrate to compare the damage chemistries induced by CAL γ_1^I and CAL θ_1^I . The 5'-[^{32}P] end-labeled fragment was treated with 0.4 μM CAL γ_1^I and 2 μM CAL θ_1^I (see Figure 2.5). The 0.4 μM CAL γ_1^I reaction resulted in a 57% production of 3'-PG which is identical to that observed in the 0.3 μM CAL γ_1^I -treated 20-mer. There was no damage in the sample which was treated with 2.0 μM CAL θ_1^I .

However, upon treatment with hydrazine, there was evidence for the 3'-phosphate-ended fragment and the 3'-phosphopyridazine. This suggests that the drug is activated by putrescine. To further assess the role of both GSH and hydrazine on the C4'-chemistries observed with CAL γ_1^I and CAL θ_1^I , the experiment was repeated with additional controls as described in the Results section.

As shown in Figure 2.6, there was no damage in the 2 μ M CAL θ_1^I -treated sample in the presence of GSH or both GSH and Glu-Gly. However, treatment with Glu-Gly resulted in the formation of putative 3'-PG. Subsequent reactions with hydrazine led to a product that migrated as expected for the 3'-phosphopyridazine. There was no reaction in the CAL γ_1^I -treated samples in the presence of Glu-Gly as expected since thiol activation is required. This result confirms that Gly-Gly serves merely as an ionic strength control in these reactions and does not participate in the activation of the drug.

In conclusion, it appears as if the presence of GSH in the CAL θ_1^I -treated samples causes a reduction in the formation of 3'-PG. This is evident because putative 3'-phosphate and 3'-PG ended fragments were only seen in the unmodified CAL θ_1^I treated samples in the presence of Glu-Gly. As mentioned in the Results section, the pH of the GSH and Glu-Gly solutions were both at 7.5, therefore pH variations in these solutions cannot explain the observed results. Further study is required to definitively determine the role that GSH plays in the C4'-chemistry mediated by CAL θ_1^I .

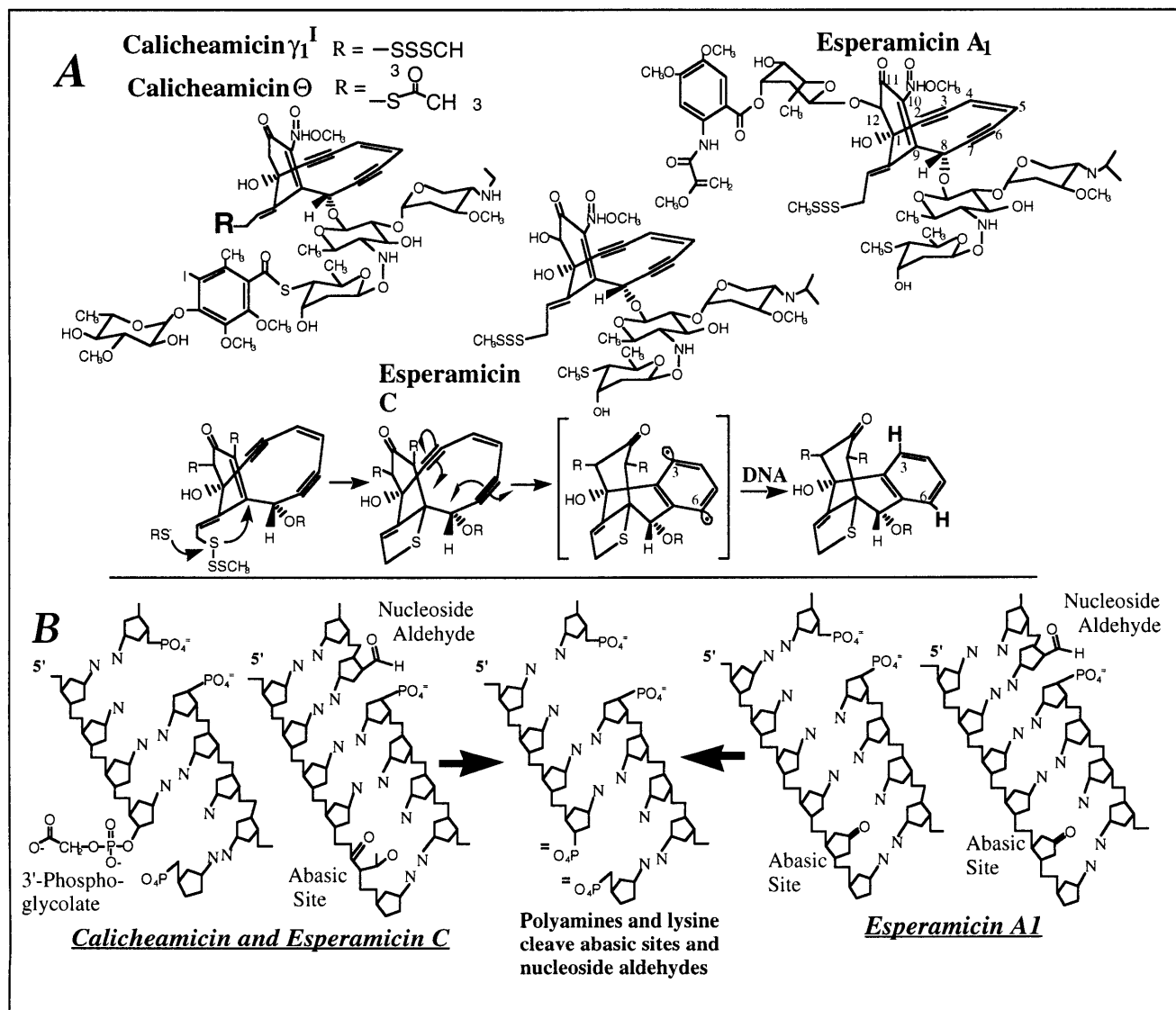


Figure 2.1. Structure, activation, and DNA damage produced by Esperamicin A₁ and Calicheamicins γ_1^I and θ_1^I

5'-GCTGATGATCCTAGACTGCC-3'
CAL3

5'-GGCAGTCTAGGATCATCAGC-3'
CAL4

Figure 2.2. Sequences of 20-mer oligonucleotides CAL3 and CAL4

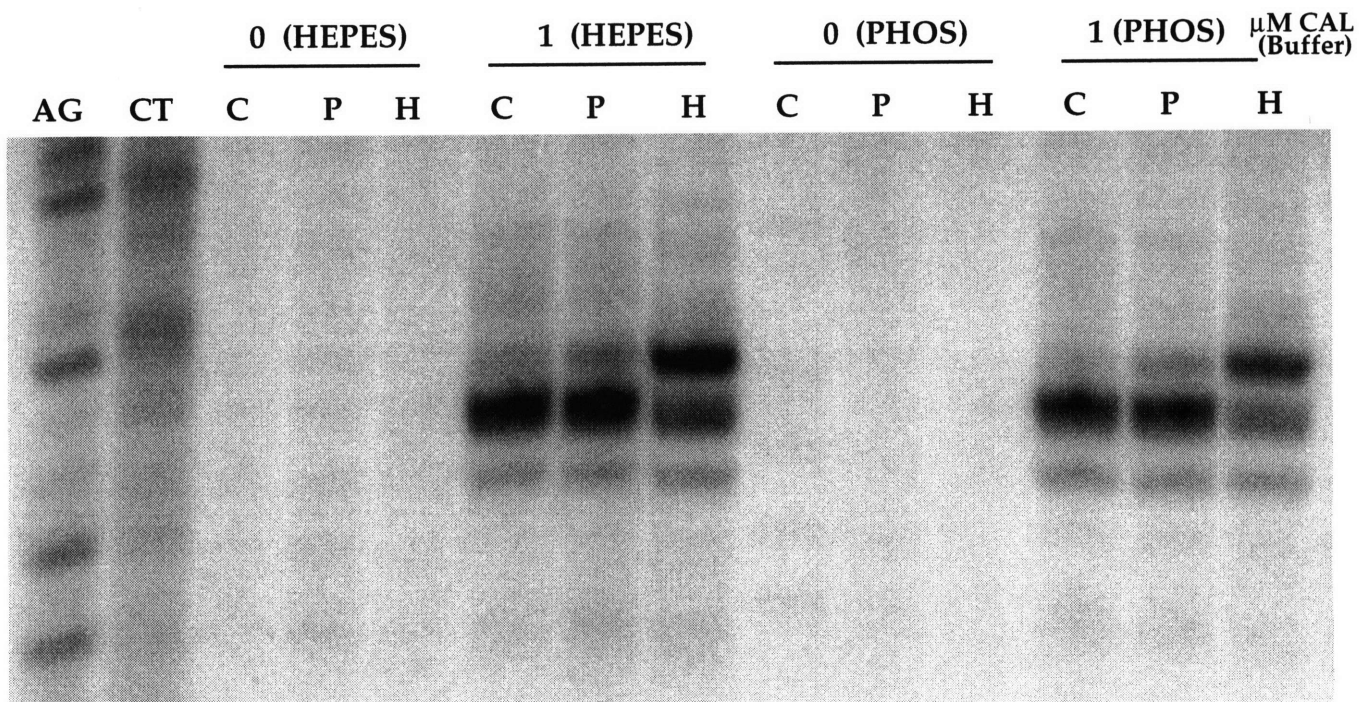


Figure 2.3. Effect of buffer composition on the distribution of CAL γ_1^I mediated C4'-chemistry. The 5'-[32 P] end labeled 20-mer (CAL34) was reacted with 1 mM CAL γ_1^I in the presence of HEPES and phosphate buffer. The oxidized DNA was subsequently left untreated (C), reacted with putrescine (P) or hydrazine(H) in order to compare the chemistries at the 3' end (against 3'-phosphate-ended Maxam-Gilbert sequencing standards indicated as AG and CT).

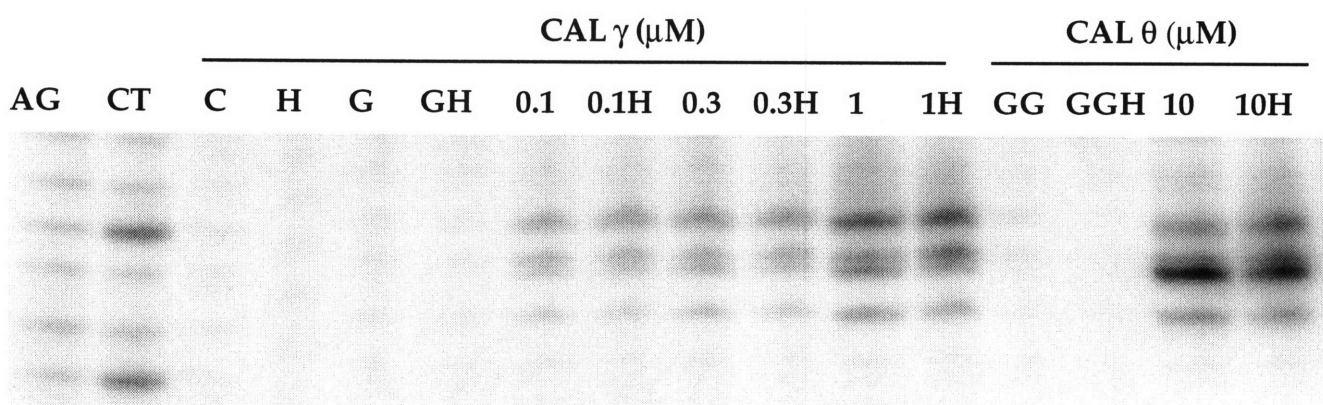


Figure 2.4. Comparison of the C4'-chemistries induced by CAL γ_1^I and CAL θ_1^I . The 5'-[32 P]-end labeled 20-mer CAL34) was treated with a range of CAL γ_1^I doses (0-1 μ M) and 10 μ M CAL θ_1^I . Following oxidation of the oligomer, the samples were either left untreated (C) or reacted with hydrazine (H) to form the 3'-phosphopyridazine of the 4'-keto 1'-aldehyde abasic site. The GSH (G) and Glu-Gly (GG) controls were also reacted with hydrazine. The Maxam-Gilbert sequencing standards (AG and CT) served as controls of 3'-phosphate-ended fragmentation products.

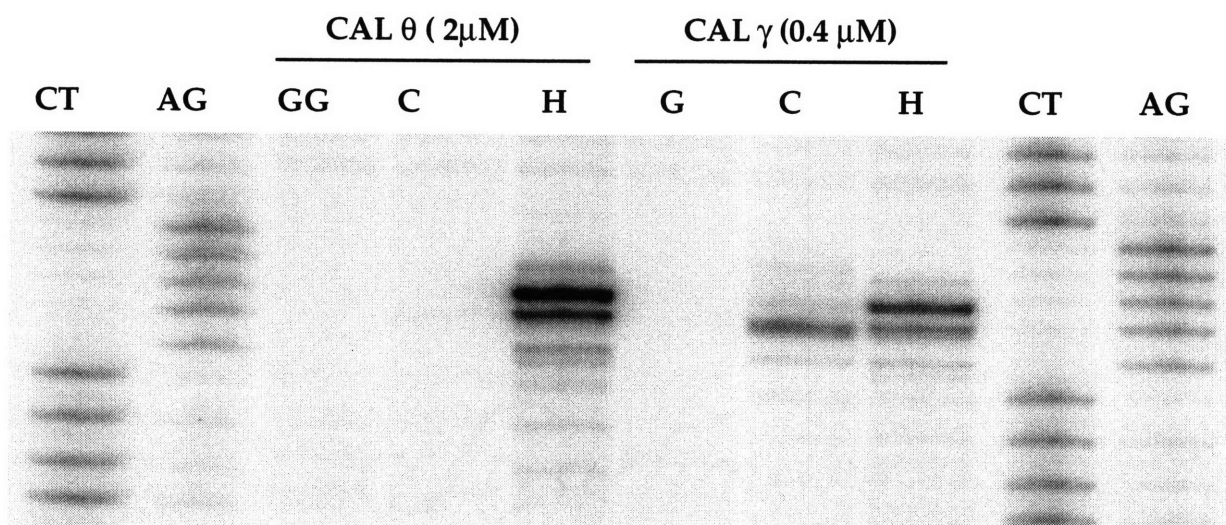


Figure 2.5. Treatment of the *Hind* III/*Pvu* II pUC19 fragment with CAL γ_1 and CAL θ_1 . The 5' 32 P end-labeled fragment was reacted with 0.4 mM CAL γ_1 and 2 mM CAL θ_1 . Following oxidation of the DNA, the samples were either left untreated (C) or reacted with hydrazine (H) to form the 3'-phosphopyridizine of the 4'-keto 1'-aldehyde abasic site. GSH (G) and Glu-Gly (GG) were added to the DNA (without drug) and run on the gel to serve as a controls of background damage. The Maxam-Gilbert sequencing standards (AG and CT) served as controls of 3'-phosphate-ended fragmentation products.

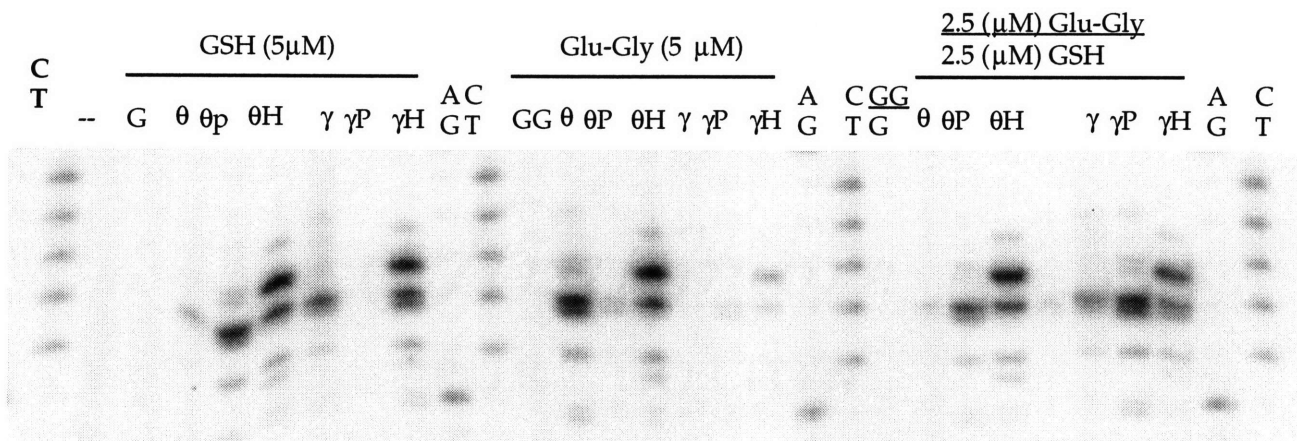


Figure 2.6. Apparent activation of CAL θ_1^I by hydrazine and putrescine. The 5' 32 P end labeled *Hind* III/*Pvu* II pUC19 fragment was treated with CAL θ_1^I in three sets of reactions: (1) with GSH (G) (2) with Glu-Gly (GG) and (3) with both GSH and Glu-Gly. After these reactions, the samples were treated with putrescine and hydrazine as previously described. Lanes AG and CT correspond to Maxam-Gilbert sequencing standards which serve as controls for 3'-phosphate-ended fragmentation.

Chapter III.
Quantitation of 8-Oxoguanine and Strand Breaks
Produced by Four Oxidizing Agents

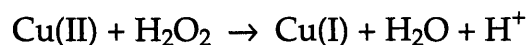
Introduction

DNA damage resulting from exposure to reactive oxygen species plays a role in a variety of biological processes such as mutagenesis, aging and carcinogenesis [3,4]. Reactive oxygen species arise in normal cellular processes such as metabolism and inflammation, and are generated during exposure to environmental chemicals, γ -radiation and transition metals [1,3,91,92]. The variety of chemistries associated with different oxidants suggests that each will produce a unique spectrum of DNA lesions consisting of single and double strand breaks, modified bases, abasic sites, and DNA-protein cross-links [93]. As part of an effort to relate genotoxin chemistry to DNA damage, this study was undertaken to define the relative quantities of two different DNA lesions, strand breaks and 8-oxoguanine (8-oxoG), produced by four oxidizing agents: peroxyxynitrite, Cu(II)/H₂O₂, Fe(II)-EDTA/H₂O₂, and γ -radiation.

Each of these oxidants produces DNA damage by a different mechanism. Peroxyxynitrite and its conjugate acid, peroxyxynitrous acid (ONOOH), are highly reactive at physiological pH and are capable of oxidizing a variety of biomolecules, including thiols, DNA and lipids [51]. Peroxyxynitrite and SIN-1, a compound that simultaneously generates nitric oxide and superoxide [94], both produce 8-oxoG [94,95] and strand breaks in DNA [40,94], and it is possible that the formation of 8-oxoG in activated macrophages relates to production of peroxyxynitrite [96]. However, peroxyxynitrite appears to have a different reactivity than the hydroxyl radical [51], which may be evident in the spectrum of DNA damage products that it produces.

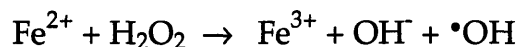
Copper (Cu) is a biologically important metal that appears to play a role in organization of the nuclear matrix [97]. In the presence of hydrogen peroxide, copper(II) has been proposed to generate hydroxyl radical-like

species through the Fenton-type reactions [98,99]:



The preferential binding of both Cu(I) and Cu(II) to the N7 of guanine may account for the high level of 8-oxoG produced by Cu and hydrogen peroxide [100-102] and for the occurrence of Cu-mediated damage [13,103] and mutation at runs of guanine [104,105]. This binding mode may also affect the relative proportions of strand breaks and 8-oxoG produced by Cu.

Iron (Fe) is another physiologically important metal that has been proposed to act as a catalyst for the formation of hydroxyl radical from hydrogen peroxide by the Fenton reaction [106]:



Of particular interest is the EDTA complex of Fe(II) that, in the presence of hydrogen peroxide, has been shown to generate a hydroxyl radical-like species [107,108]. Unlike free Cu and Fe, the negative charge of the Fe(II)-EDTA complex likely prevents it from binding to DNA phosphates or bases [108,109]. The resulting hydroxyl radical-like species may exist in the fluid phase, which could account for the lack of base sequence-selectivity of strand breaks generated by the complex [108,109]. In this respect, Fe(II)-EDTA may be similar to γ -radiation in its DNA damage spectrum.

In addition to a minor contribution from direct interaction with DNA, γ -radiation reacts with water to produce hydroxyl radicals and hydrogen atoms by homolytic fission of oxygen-hydrogen bonds and to produce hydrated electrons [29]. As with Fe(II)-EDTA, γ -radiation-induced hydroxyl radical can react with both deoxyribose and bases in DNA to produce damage that includes strand breaks [110,111] and 8-oxoG [112].

While these four agents all produce strand breaks and 8-oxoG, they do

so by different mechanisms and in different locations in DNA. Even the final common damage products can arise by different mechanisms. This is most evident with oxidation of deoxyribose, which can result in both direct strand breaks and oxidized abasic sites. For example, γ -radiation and the antibiotics neocarzinostatin and bleomycin all produce radicals that can abstract a hydrogen atom from the C4'-position of deoxyribose [12,15,29,93]. The resulting C4'-radical can undergo further reactions to form either a strand break with a 3'-phosphoglycolate residue or a 4'-keto-1'-aldehyde abasic site that leaves the sugar-phosphate backbone intact [12,15,29,93]. However, formation of the ketoaldehyde abasic site is oxygen-independent with bleomycin [12] and oxygen-dependent with neocarzinostatin and γ -radiation [15,29]. Identical products can thus arise by different mechanisms depending on the chemistry of the oxidizing agent.

With regard to base damage, our studies focus on 8-oxoG, which was identified in 1984 by Kasai and Nishimura [113]. The development of a sensitive analytical technique involving HPLC with electrochemical detection [114] has made 8-oxoG an attractive marker for monitoring DNA damage in studies with various oxidizing agents [115]. The role of 8-oxoG in mutagenesis and carcinogenesis has been widely investigated [116] and many studies have shown a correlation between the formation of 8-oxoG and carcinogenesis [117]. Oxygen radicals appear to mediate the formation of 8-oxoG in DNA through a reaction involving the addition of hydroxyl radical to the C-8 of guanine [117]. This is followed by the subsequent loss of a hydrogen atom, or by a one-electron oxidation of the C-8 position and subsequent addition of water [116], which may again depend on the chemistry of the oxidizing agent.

In an effort to understand the relationship between the reactivity of a

genotoxin and the DNA damage it produces, strand breaks and 8-oxoG for peroxyxynitrite, Cu(II)/H₂O₂, Fe(II)-EDTA/H₂O₂, and γ -radiation were quantified at exposure levels approaching physiological relevance. We found that the ratio of 8-oxoG to strand breaks was not the same for different oxygen radical generating systems, and the relevant proportions of 8-oxoG and strand breaks varied as a function of concentration for certain oxidizing agents. These results are discussed in light of other investigations of oxidative DNA damage spectra and the current models for the mechanism of action of the four oxidizing agents.

Material and Methods

Chemicals. 2-Amino-6,8-dihydroxypurine (8-oxoG) was obtained from Chemical Dynamics Corp. Plasmid pUC19 (2686 base pairs) was obtained from New England Biolabs. Cyanogen bromide-activated Sepharose 4B, N²-methyl guanosine, diethylaminetriaminepentaacetic acid (DETAPAC), phosphate buffered saline (PBS), and putrescine were purchased from Sigma Chemical Co. Cupric chloride, ethylenediaminetetraacetic acid (EDTA), potassium phosphate (mono and dibasic), agarose, and acetonitrile were obtained from Malinckrodt, hydrogen peroxide (30%) and ammonium acetate were purchased from Fisher Scientific. Dimethyl Sulfoxide (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. [γ -³²P]ATP (150 mCi/mL) was obtained from Amersham. [³H]8-oxoG was generously provided by Dr. William Boadi (Division of Toxicology, Massachusetts Institute of Technology). Plasmid Giga prep purification kits were purchased from Qiagen.

Preparation and purification of pUC19 plasmid DNA. Cultures of DH5 α *Escherichia coli* cells were transformed with pUC19 plasmid and plated on LB agar plates containing 50 μ g/mL ampicillin. Colonies were then isolated from the plates and grown to late log phase in standard LB medium containing 100 μ g/mL ampicillin [61]. Plasmid DNA was isolated using the Qiagen Giga Plasmid/Cosmid Purification kit according to protocols established by the manufacturer. After washing the DNA pellet with 80% ethanol, the solvent was lyophilized and the plasmid DNA was dissolved in 50 mM potassium phosphate, pH 7.4 (treated with Chelex 100 resin).

The plasmid DNA was then dialyzed against 50 mM potassium

phosphate containing 1 mM DETAPAC for 12 hr at 4 °C to remove trace metals and then against 50 mM potassium phosphate for an additional 12 hr. at 4 °C. The concentration of the dialyzed plasmid was then determine by a Hoechst dye assay using a Sequoia-Turner Model 450 fluorometer. To minimize nicking of the plasmid it was stored in 200- μ g aliquots at -80 °C.

Synthesis of Peroxynitrite. A low ionic strength, high pH solution of peroxynitrite anion (ONOO⁻) was synthesized using the ozonolysis of sodium azide method described by Pryor *et. al.* [108]. The synthesis was achieved using a Welsbach ozonator set at an oxygen pressure of 7 psi, a voltage of 60 V and an ozone pressure ranging between 1.5-2.0 psi. A gas stream from the ozonator was bubbled through a glass-frit into 100 mL of a 100 mM sodium azide solution in deionized H₂O (the pH was previously adjusted to 12 with 1 N NaOH) chilled to 0-4 °C in ice water for 90 min.

To optimize the yield of ONOO⁻, aliquots (300 μ L) of the ozonated azide solution were removed every 10 min and the concentration of ONOO⁻ in these fractions was determined spectrophotometrically (see Figure 3.1) in 0.1 N NaOH by measuring the absorbance at 302 nm using an extinction coefficient of 1670 M⁻¹ cm⁻¹ [118]. The peroxynitrite was further characterized for its ability to cause the nitration of L-tyrosine using a method described by Beckman *et. al.* [119] as modified by Pryor *et. al.* [120].

Oxidation of pUC19 Plasmid DNA. γ -Radiation, Fe(II)-EDTA, peroxynitrite and Cu/H₂O₂ treatment of plasmid was performed under single-hit conditions in which each DNA molecule received less that or equal to one damage event per reaction. This level of damage was chosen to insure the physiological relevance of the study and reliability of strand break quantitation assay. For each oxidizing agent, the plasmid concentration was 30 μ g/mL in Chelex 100 treated 50 mM potassium phosphate, pH 7.4, and all

reactions were done at ambient temperature for 30 min. The ferrous ammonium sulfate, cupric chloride, EDTA and hydrogen peroxide solutions were prepared immediately prior to each treatment.

Reactions with Fe(II)-EDTA were performed at Fe(II) concentrations of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 5, and 10 μM (Fe(II) solutions were prepared deionized H_2O with a 2-fold molar excess of EDTA and 1 mM H_2O_2 (prepared in Chelex 100 treated phosphate buffer) for each treatment. Control studies revealed that the damage reaction was complete at the end of the 30 min incubation and that there was no further damage during the subsequent dialysis for 8-oxoG analysis (*vide infra*; data not shown). Reactions with Cu(II) were performed at CuCl_2 concentrations of 0, 5, 7.5, 10, 12.5, 15, 20, 25 and 30 μM (prepared in deionized H_2O) with 1 mM H_2O_2 . The reaction was stopped by adding EDTA to a final concentration of 1 mM EDTA. Previous studies have revealed that EDTA abolishes Cu(II)/ H_2O_2 induced oxidative damage [121]. The peroxyxynitrite reactions were performed at concentrations of 0, 0.25, 0.5, 1.0, 5, 10, 15, 20, 25 and 50 mM. Peroxyxynitrite has a half-life of ~ 1 sec at pH 7.4 and the nitrate rearrangement products have been shown to be unreactive [50]. Lastly, the plasmid was also reacted with γ -radiation at 0, 0.125, 0.375, 0.5, 1, 5, and 10 Gy exposures using a ^{60}Co γ source at a dose rate of 4 Gy/min. The radiation experiments were performed in either air- or N_2O -saturated phosphate buffer.

Preparation of random primer probe. A [^{32}P] labeled random primer probe using a modified version of the technique described by Feinberg *et al.* [122]. Initially, 50 ng of *Dde* I-digested pUC19 and 1.25 units of a random hexamer primer in a total volume of 3 μL were boiled for 3 minutes and immediately placed on ice. To this reaction mixture was added 10 μg BSA, 10 μL of 2.5x reaction buffer (50 μM dCTP, 50 μM dGTP, 50 μM dTTP, 50 μM

β -mercaptoethanol, 12.5 mM MgCl₂, 125 mM Tris, 500 mM HEPES pH 6.6), 100 μ Ci [α -³²P] dATP and 5 units Klenow fragment in a total volume of 25 μ L and the solution was incubated at 37 °C. After the incubation period, the reaction was stopped by adding EDTA to a final concentration of 10 mM and the Klenow fragment was inactivated by heating at 68 °C for 1 min. The reaction mixture was then extracted with phenol:chloroform:isoamyl alcohol and passed over a G50-sephadex spin column to remove unincorporated radionucleotides.

Quantitation of strand breaks. Quantitation of damage in plasmids treated with γ -Radiation, Fe(II)-EDTA, peroxyntirite and Cu(II)/H₂O₂ was accomplished using a method similar to one described previously [37]. The method involved damaging the plasmid under single-hit conditions, assuming a Poisson distribution, and calculating the level of damage based on the formation of single-strand nicked plasmid (form II) from the >95% supercoiled (form I) starting material. After treatment with γ -radiation, Fe(II)-EDTA, peroxyntirite and Cu(II)/ H₂O₂, a fraction of the plasmid DNA was treated with putrescine (100 mM, pH 7, 1 h 37 °C) to cleave abasic site and express the damage as nicked, form II plasmid.

The damaged plasmid (300 ng) was then loaded on a 1% agarose gel (Tris-borate-EDTA), and the plasmid topoisomers (form I and II) were resolved at 3V/cm for 3-4 hr. The gel was then dried under vacuum and probed with the radiolabeled pUC19 random primer probe using hybridized techniques [61]. The plasmid topoisomers were quantified by exposing the radiolabeled gel to a phosphor plate and subsequently analyzing the image using the ImageQuant software on a Molecular Dynamic PhosphorImager (see figures 3.2, 3.3 and 3.4 for resolution of damaged plasmids on 1% agarose gels).

Quantitation of 8-oxoguanine The base damage product 8-oxoguanine (8-oxoG) was quantitated by Laura Kennedy as described elsewhere [41]. Briefly, monoclonal antibodies specific to 8-oxoG were produced as described previously by Ravanat *et. al.* [123]. These monoclonal antibodies were subsequently coupled to CNBr-activated Sepharose 4B [124]. Immediately prior to use in the immunoaffinity columns, the antibody-modified gel was washed with 50% DMSO to remove any bound 8oxoG. The gel was then washed with an equal volume of water. The binding efficiency of the antibody-modified gel was determined to be 75-80% using [³H]8oxoG. *N*²-methyl-8-oxoguanosine was then synthesized [41] to serve as an internal control for the amount of 8-oxoG which eluted from the immunoaffinity columns.

For subsequent analysis of 8-oxoG, the γ -radiation, Fe(II)-EDTA, peroxyxynitrite and Cu/H₂O₂ treated DNA was dialyzed against deionized water for 16-20 hr at 4 °C. The absence of reducing agents, the low temperature, and the fact that the reaction were completed or stopped prior to dialysis rule out the possibility of significant adventitious DNA damage during the dialysis. Following dialysis, the concentration of DNA in the solution was determined by UV at 260 nm (1 OD = 50 μ g), and 50 μ g was aliquotted in triplicate into screw-cap vials. The DNA was then dried under vacuum and hydrolyzed in a final concentration of 60% formic acid at 100 °C for 1 hr. The formic acid was then removed by centrifugation under vacuum and the hydrolyzed DNA was resuspended in 0.5 mL PBS, loaded on the immunoaffinity columns, and the bound 8-oxoG was eluted with 1.0 mL of methanol. After incubation at -20 °C for 30 min and the addition of 150 μ L of *N*²-methyl-8-oxoguanosine solution (10 pg/ μ L), the eluent was then dried by centrifugation under vacuum in preparation for quantification of 8-oxoG by HPLC.

HPLC System. The HPLC 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 μ m 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.

Quantification of 8-oxoG by HPLC. Following immunopurification of 8-oxoG, the dried samples were resuspended in 50 μ L of the HPLC mobile phase. The samples were then injected onto the Supelco LC-18-DB 5 μ m column, and the levels of 8-oxoguanosine were calculated by integration of the areas under the 8-oxoG and *N*²-Methyl-8-oxoG peaks in the resulting chromatograms. From a calibration curve relating known amounts of 8-oxoG to *N*²-Methyl-8-oxoG, the ratio of 8-oxoG/ *N*²-Methyl-8-oxoG was the converted to the number of 8-oxoG per 10⁶ bases. The background level of 8-oxoG was subsequently determined to be ~5 residues per 10⁶ bases. This value was subtracted from the 8-oxoG levels induced by the oxidizing agents.

Results

γ -Radiation [110-112], Fe(II)-EDTA/H₂O₂ [108,109,125], Cu(II)/H₂O₂ [100,126,127], and peroxyxynitrite [40,94] all produce strand breaks and 8-oxoG but apparently by different mechanisms. Therefore, we used these four oxidizing agents to compare the relative quantities of strand breaks and 8-oxoG produced during oxidative DNA damage.

γ -Radiation-induced Damage. The quantities of both 8-oxoG and strand breaks were found to increase with increasing doses of radiation (Figure 3.5). For all of the oxidizing agents, the formation of strand breaks was studied in the range of "single-hit conditions" which corresponds to ~30-40 direct strand breaks in 10⁶ bases of pUC19 DNA according to a Poisson distribution [20]. Above this level of damage, the number of strand breaks is underestimated because additional nicks in an already nicked plasmid cannot be detected by topoisomer analysis. Under single-hit conditions, the increase in strand breaks and 8-oxoG produced by γ -radiation was nearly linear. However, the amount of 8-oxoG formed was significantly less than the number of strand breaks. The data reported are for air-saturated solutions since only slightly higher levels of strand breaks were observed under N₂O, and no significant effect was observed for 8-oxoG (data not shown).

Because oxidation of deoxyribose results in both strand breaks and abasic sites, we sought to quantify abasic site formation by converting the lesions to strand breaks with putrescine. This agent has been shown to cleave virtually all types of induced abasic sites in DNA [9,20,70,128]. Interestingly, putrescine treatment did not increase the number of apparent strand breaks by more than 5% for radiation and the other oxidizing agents examined in these studies (data not shown). This observation suggests that, under our conditions, abasic sites represent a relatively small component of the total

DNA damage. Since abasic sites can also arise from loss of oxidized bases, the small effect of putrescine treatment also indicates that putrescine did not induce significant abasic site formation by reaction with bases lesions produced by the four oxidizing agents.

Iron(II)-EDTA-induced Damage. As observed with γ -radiation, there was a linear increase in both strand break and 8-oxoG formation upon treatment with Fe(II)-EDTA/H₂O₂ (Figure 3.6). The relative levels of 8-oxoG and strand breaks were similar to those observed with γ -radiation, which is consistent with a hydroxyl radical-like DNA damaging species.

Copper(II)-induced Damage. The levels of strand breaks and 8-oxoG produced by Cu(II)/H₂O₂ were different from those seen with γ -radiation and Fe(II)-EDTA/H₂O₂ (Figure 3.7). Most significantly, the number of strand breaks rose sharply between 7.5 and 12.5 mM Cu(II) while the quantity of 8-oxoG increased gradually. The different shapes of the strand break and 8-oxoG curves is consistent with the observations of Drouin *et. al.* in their studies of strand breaks and base damage in isolated genomic DNA [129] and suggests that strand breaks and 8-oxoG are produced by different mechanisms. The levels of 8-oxoG were higher than those arising from γ -radiation and Fe(II)-EDTA, which is consistent with a site specific reaction involving copper bound to the N7 position of guanine [101,102].

Peroxynitrite-induced Damage. As shown in Figure 3.8, the levels of 8-oxoG were higher than those observed for γ -radiation and Fe(II)-EDTA, indicating that the reactive oxygen species produced by peroxynitrite reacts with a different selectivity than the hydroxyl radical produced by radiation or the hydroxyl radical-like species associated with Fe(II)-EDTA. Additionally, the shape of the strand break curve at lower concentrations of peroxynitrite suggests that the reaction is biphasic in nature.

As mentioned earlier, there was little increase in strand breaks following putrescine treatment of the peroxynitrite-damaged plasmid DNA. This is important in light of the observation by Yermilov *et. al.* that peroxynitrite produces higher levels of 8-nitroguanine than 8-oxoG and that 8-nitroguanine undergoes depurination with a half-life of ~4 hr at 20 °C [95]. The lack of putrescine effect indicates that putrescine did not react with either 8-oxoG or 8-nitroguanine to produce strand breaks and that depurination did not occur to any appreciable extent during DNA processing, since putrescine would have converted the abasic sites to strand breaks.

Discussion

Oxidative DNA damage resulting from exposure to reactive oxygen species is believed to play a significant role in mutagenesis, aging and carcinogenesis [3,4]. In an ongoing effort to relate the chemistry of genotoxins to the spectrum of DNA lesions they produce, we have examined the quantities of 8-oxoG and strand breaks produced by four different oxidizing agents. We have demonstrated that the relative levels of 8-oxoG and strand breaks can vary by more than an order of magnitude and that they can vary between different concentrations of a single agent as shown most dramatically for Cu(II)/H₂O₂. These results indicate the importance of understanding how the chemistry of the oxidizing agents contributes to the different levels and types of oxidative damage to DNA.

Both Fe(II)-EDTA/H₂O₂ and γ -radiation generate similar levels of 8-oxoG and strand breaks which is consistent with the hypothesis that chemical intermediates of similar reactivity are involved in each system. The results are also consistent with previous studies in which the sequence specificity of DNA damage produced by γ -radiation was shown to be similar to that of Fe(II)-EDTA [16,130]. In both cases, the majority of the DNA damage can be attributed to a hydroxyl radical-like species generated by the reagent. It is possible that the higher levels of strand breaks relative to 8-oxoG reflect the different fates of the initial one electron oxidation products, the accessibility of the Fe/EDTA complex to the DNA grooves, or, in the case of radiation, the higher concentration of water molecules in the solution phase compared to the grooves.

The levels of 8-oxoG relative to strand breaks were higher with Cu(II) than with Fe(II)-EDTA or γ -radiation. This difference could be due to generation of a different DNA-damaging species or to the DNA binding

specificity of Cu(II). Preferential binding of Cu(II) to the N7 of guanine makes the C-8 position susceptible to attack by hydroxyl radical or some other Cu-induced oxidizing species.

Unlike the smooth rise in 8-oxoG formation, there was a sharp increase in strand breaks between 7.5 and 12.5 mM Cu(II) (Figure 3.7). This sudden transition was also observed with the Cu(II)/H₂O₂/ascorbate system, which indicates that it is not unique to the Cu(II)/H₂O₂ reagent (data not shown). A sharp increase in strand breaks and a more gradual increase in base lesions was also observed by Drouin *et al.* in their studies of DNA lesions produced by Cu(II)/H₂O₂/ascorbate [129]. The results of both studies are consistent with a model in which Cu-induced strand breaks and 8-oxoG are formed by different mechanisms or DNA binding modes.

While the model that Cu-induced strand breaks and base damage occur by different mechanisms is in contrast to the model proposed recently by Toyokuni and Sagripanti [131], data from the two studies is in fairly good agreement. At similar ratios of Cu to DNA, we observe a ratio of strand breaks to 8-oxoG of ~3 *versus* a ratio of ~2 determined by Toyokuni and Sagripanti [131]. In their studies, Toyokuni and Sagripanti held the Cu(II) concentration constant and varied the level of H₂O₂ [131]. Since both base damage and strand breaks produced by Cu have been shown to increase as a function of increasing H₂O₂ concentration [129,132], their observation of a linear relationship between strand breaks and 8-oxoG under these conditions is consistent with the model that Drouin *et al.* [129] and we have proposed: the ratio of strand breaks to 8-oxoG remains constant at one Cu concentration with both lesions varying in parallel as a function of the H₂O₂ concentration.

Peroxynitrite also generated higher levels of 8-oxoG relative to strand breaks than did Fe(II)-EDTA and γ -radiation. This is consistent with a DNA-

damaging species other than hydroxyl radical such as a high energy form of peroxyxynitrous acid [51]. On the basis of studies with hydroxyl radical scavengers, the high energy intermediate is believed to be less reactive and more selective than the hydroxyl radical [40,51]. Our data lend further support to this hypothesis.

There are several interesting features of the peroxyxynitrite concentration-damage profile shown in Figure 3.8. First is the plateau and possibly decrease in 8-oxoG formation at high concentrations of peroxyxynitrite. Yermilov *et. al.* also observed a plateau effect with peroxyxynitrite [95], while Inoue and Kawanishi, using SIN-1, observed a decrease in 8-oxoG with increasing peroxyxynitrite concentrations [94]. These observations are all consistent with the recent studies of Uppu *et. al.* who observed that 8-oxoG is susceptible to further oxidation by peroxyxynitrite [133]. Because they were unable to detect 8-oxoG formation at high peroxyxynitrite concentrations (0.1-1 mM), Uppu *et al.* proposed that either 8-oxoG was not formed or that any 8-oxoG that is produced is rapidly oxidized by peroxyxynitrite [133]. Our results support the latter hypothesis. In addition to the use of a more sensitive 8-oxoG assay, our ability to detect 8-oxoG is likely due to that fact we performed the DNA damage reactions at lower peroxyxynitrite concentrations (0.25-100 μ M *vs.* 100-1000 μ M), which presumably reduced the second-order oxidation of 8-oxoG by peroxyxynitrite. Our results cannot be attributed to different preparations of peroxyxynitrite since we used the same procedure as Uppu *et. al.* to prepare the peroxyxynitrite [133].

The second interesting feature of peroxyxynitrite-induced DNA damage is the biphasic nature of strand break production. We are presently unable to explain this phenomenon, but it may be due to some type of competing second order reaction such as a self reaction of peroxyxynitrite.

In conclusion, we have determined that the relative quantities of sugar and base damage produced by four different oxidizing agents vary as a function of the chemistry and concentration of the agent. The results are consistent with several previous studies and extend the observations into physiologically relevant concentrations of oxidizing agents. The observations made with each agent warrant further study.

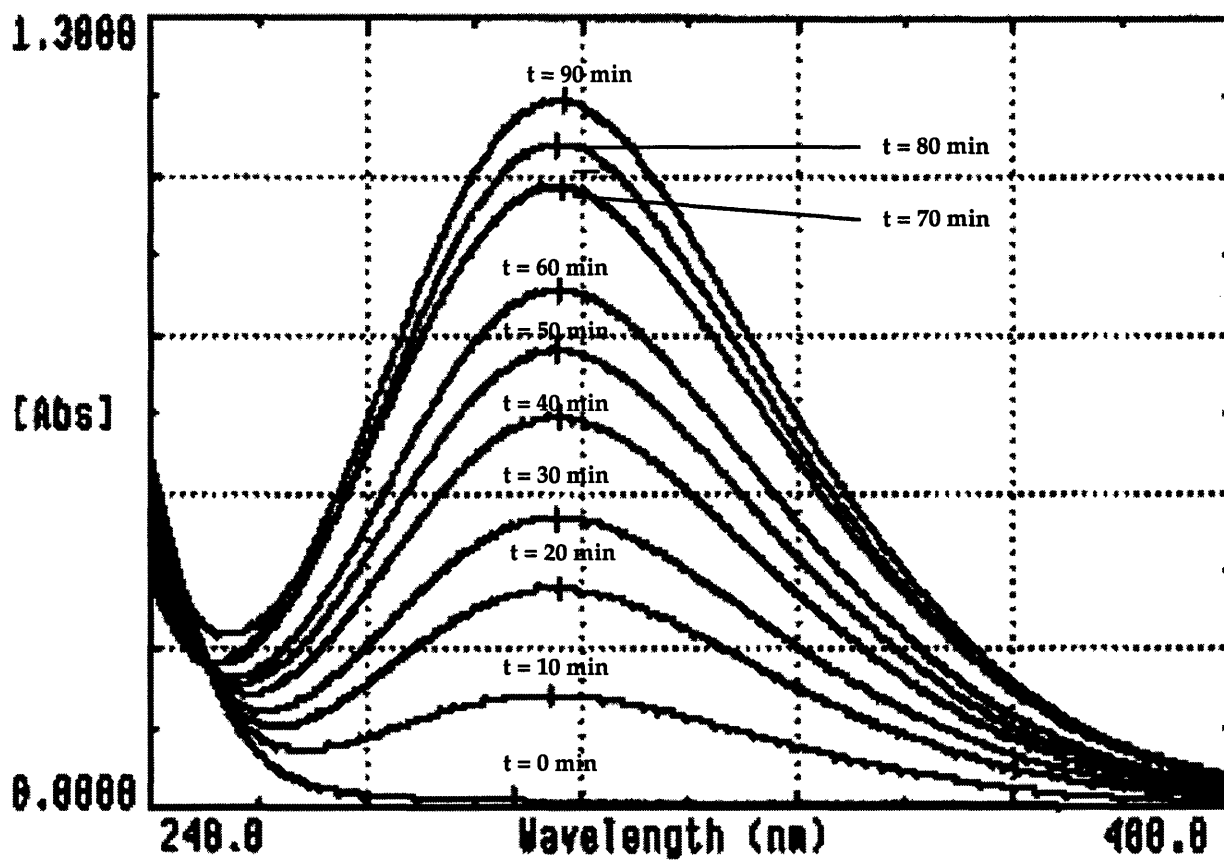


Figure 3.1. Synthesis of peroxynitrite using the ozonolysis of sodium azide method. Aliquots of the 100 mM NaN_3 solution were removed in 10 min intervals and UV scanned from 240-400 nm ($\epsilon_{302} = 1670 \text{ M}^{-1} \text{ s}^{-1}$)

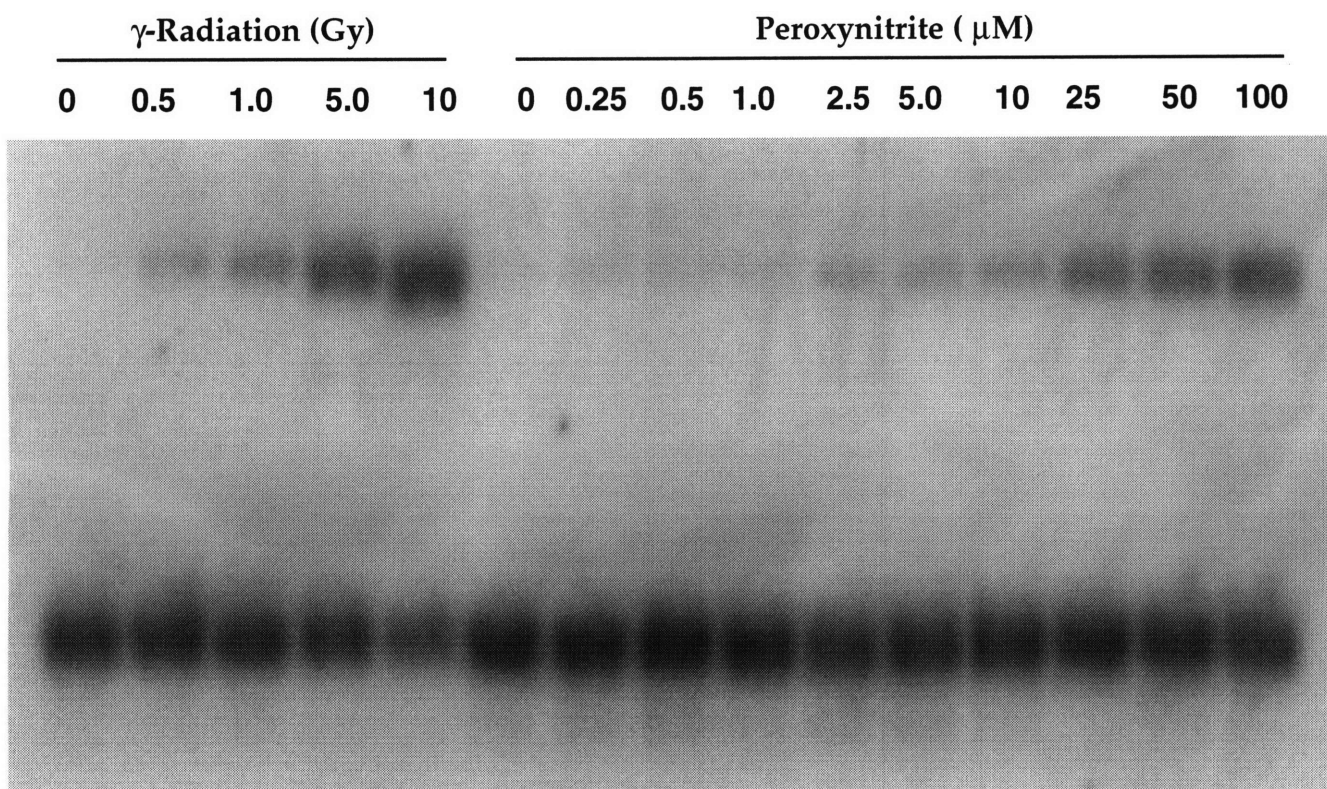


Figure 3.2. Strand breaks induced by γ -radiation and peroxynitrite. The plasmid pUC19 was reacted with γ -radiation and peroxynitrite as described the in Material and Methods section and the form II (nicked) DNA was resolved from the form I (undamaged supercoiled) DNA.

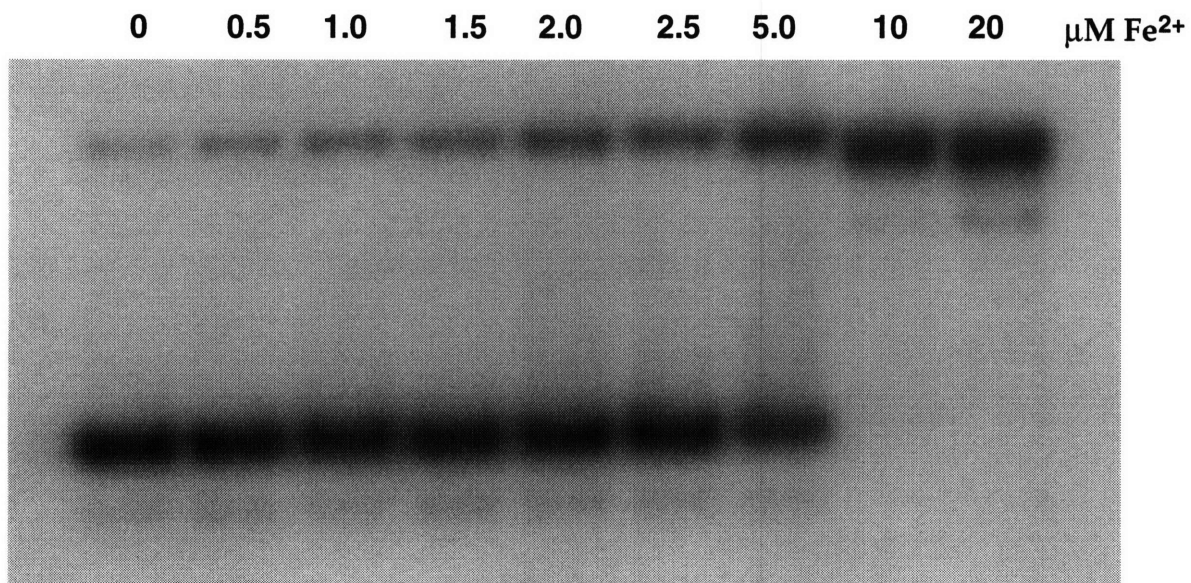


Figure 3.3. Strand breaks induced by $\text{Fe(II)/H}_2\text{O}_2$. The plasmid pUC19 was reacted with $\text{Fe(II)/H}_2\text{O}_2$ as described in the Material and Methods section and the form II (nicked) DNA was resolved from the form I (undamaged supercoiled) DNA.

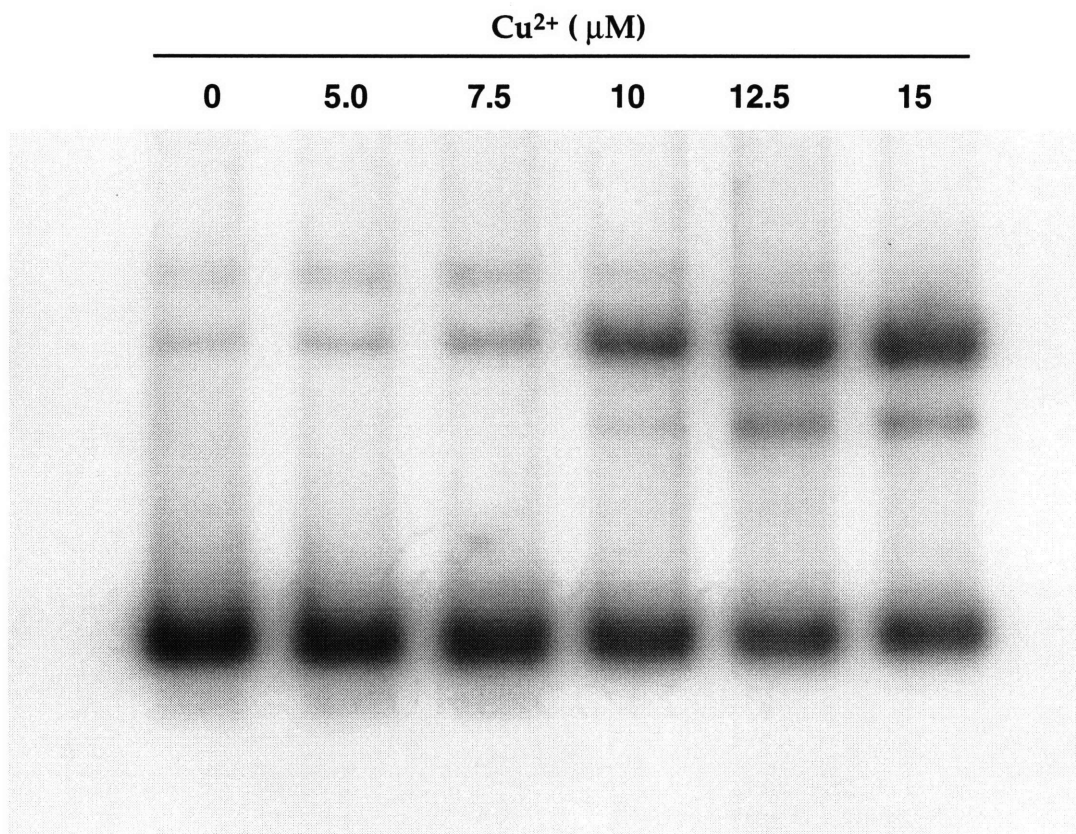


Figure 3.4. Strand breaks induced by $\text{Cu}(\text{II})/\text{H}_2\text{O}_2$. The plasmid pUC19 was reacted with $\text{Cu}(\text{II})/\text{H}_2\text{O}_2$ as described in the Material and Methods section and the form II (nicked) DNA was resolved from the form I (undamaged supercoiled) DNA.

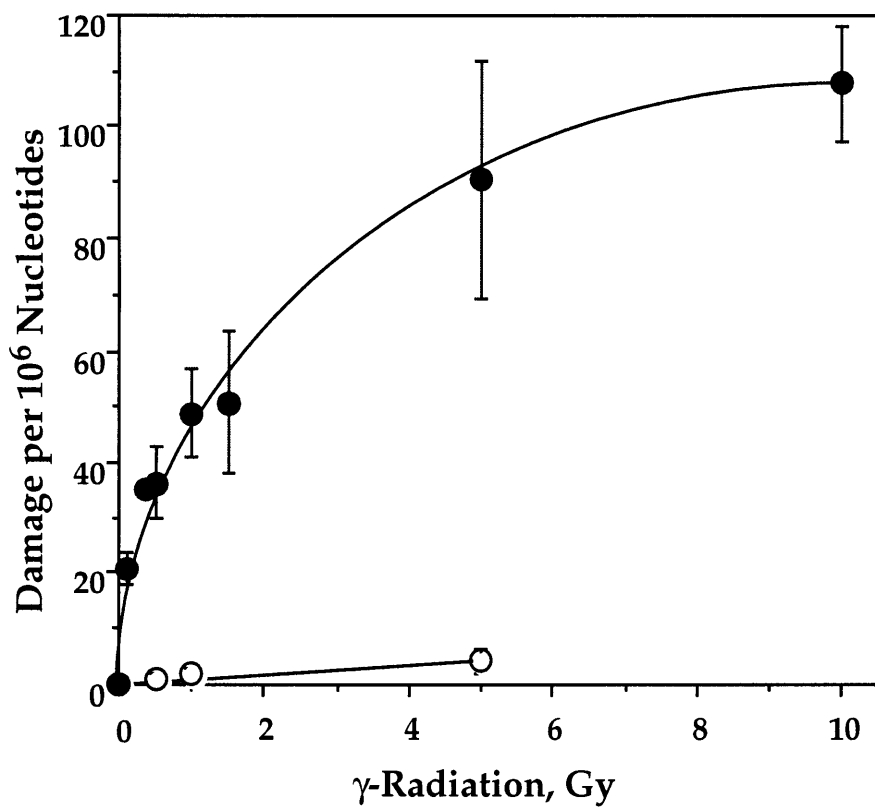


Figure 3.5. 8-oxoG (o) and strand breaks (•) produced by γ -radiation.

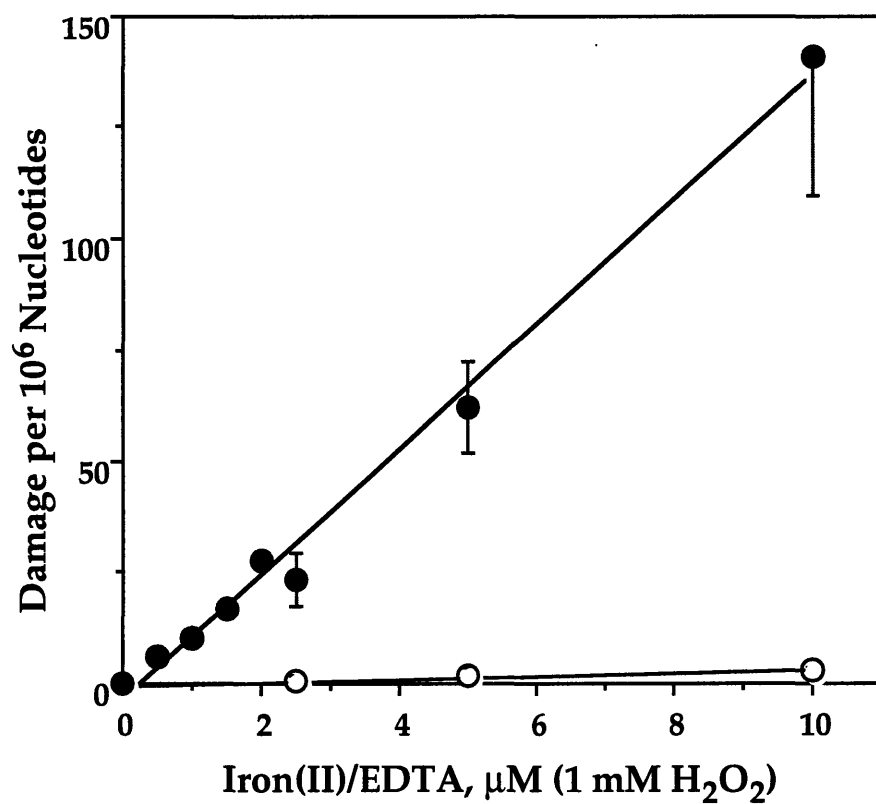


Figure 3.6. 8-oxoG (o) and strand breaks (•) produced by Fe(II)-EDTA/H₂O₂.

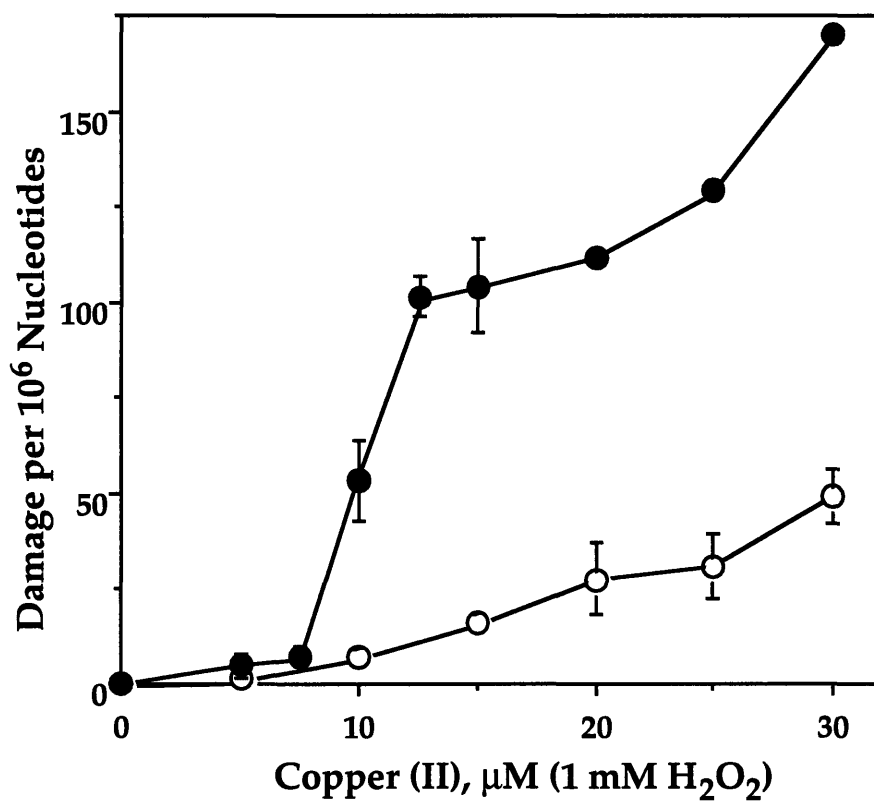


Figure 3.7. 8-oxoG (o) and strand breaks (•) produced by Cu(II)-EDTA/H₂O₂.

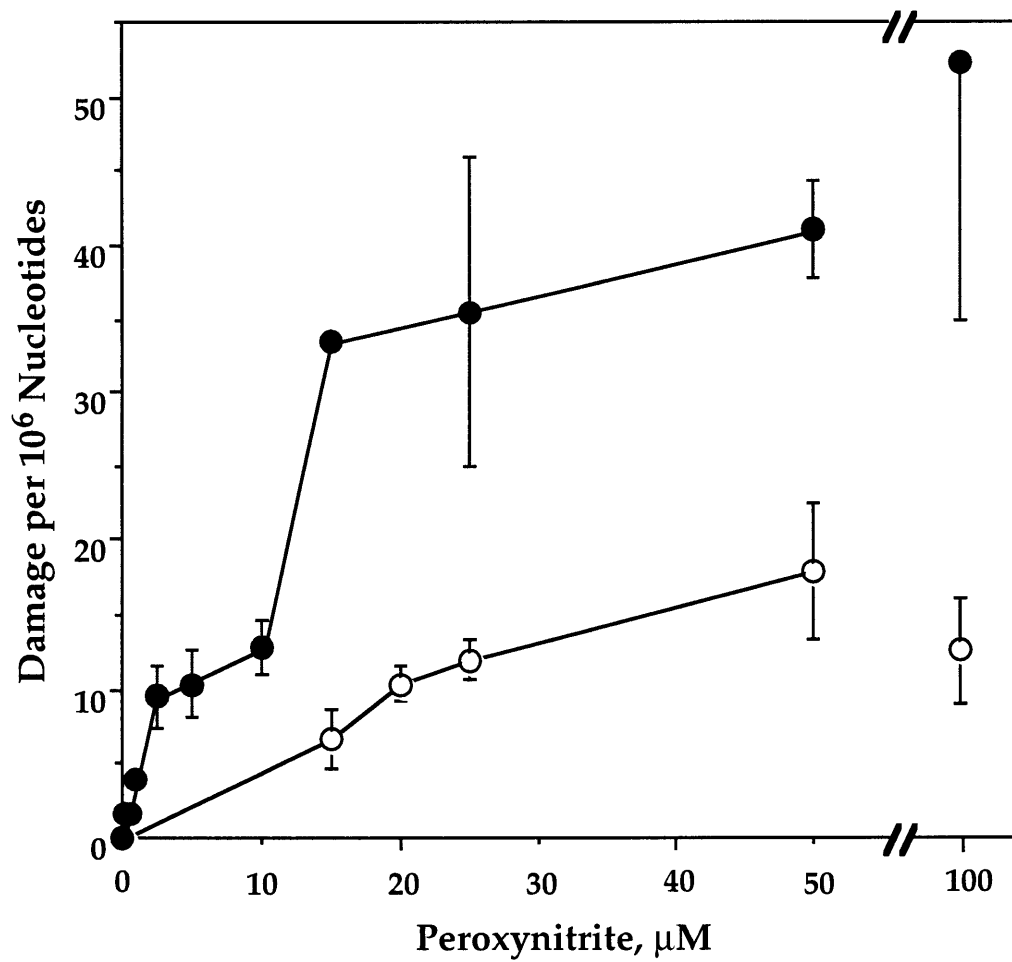


Figure 3.8. 8-oxoG (o) and strand breaks (•) produced by peroxynitrite.

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